REMARKS

Re-consideration of the above-identified application in view of the following remarks is respectfully requested.

Claims 1-14 are in this case. Claims 1-4 were withdrawn under a restriction requirement as drawn to a non-elected invention. Claims 5-14 have been rejected. Claim 5 is currently amended, claim 6 is cancelled and claim 15 is new.

35 U.S.C. § 103(a) Rejections - Altman in view of Matsumura

The Examiner has rejected claims 5-14 under 35 U.S.C. § 103(a) as being unpatentable over Altman et al. (Proc. Nat. Acad. Sci. USA; 1993, 90: 10330-10334) in view of Matsumura et al. [J. Biol. Chem.; 1992, 267 (33): 23589-23595]. The cited prior arts describe the expression of the α and β chains of the MHC class II complex in *E. coli* (Altman) or the HLA and β2 microglobulin (β2-m) polypeptides of the MHC class I complex in eukaryotic cells (Matsumura) which are completely unstable at physiological temperatures of 37 °C. Support for that can be found by the negligible yield at physiological temperatures (e.g., 37 °C) of the *in vitro* re-folded MHC class II complex in the presence of the BioMCC antigenic peptide (Figure 1b in Altman et al., 1993) and by the disappearance of the immunostaining band which corresponds to the K^b heavy chain protein at a temperature range of 37-47 °C (Figure 1c in Matsumura et al., 1992).

In sharp contrast to the references cited by the Examiner, the present invention relates to a method which enables generation of a single chain MHC class I molecule in *E. coli* which, following *in vitro* refolding in the presence of an antigenic peptide [as reflected in step (c) of claim 5], forms a functional and physiologically stable MHC class I-peptide complex (support for complex stability can be found in Figures 4, 7-10 and Example 1 of the Examples section of the present invention). Thus, the teachings of the cited prior arts do not teach, nor motivate the ordinary skilled artisan to make the present invention.

The instability of the MHC II-peptide complex produced in *E. coli* by Altman et al. is due to the production of the two independent α and β polypeptides from two

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separate clones (*i.e.*, Ec-I-E^k α and β). Thus, when such a non-covalent complex is formed, each of the complex components (e.g., the α and β polypeptides and the antigenic peptide) as well as the combined complex is more susceptible to dissociation and degradation (hence instability) rather than when a complex is formed from a single chain. In addition, it is well known that in MHC-I complexes (which are different from those used in Altman et al), the β2-m polypeptide stabilizes the groove formed by the α1-α2-α3 domains of the HLA molecule [Berko D, et al., 2005, Membrane-anchored beta 2-microglobulin stabilizes a highly receptive state of MHC class I molecules. J. Immunol. 174(4): 2116-23; Shields MJ, et al., 1998, Characterization of the interactions between MHC class I subunits: a systematic approach for the engineering of higher affinity variants of beta 2-microglobulin. J. Immunol. 160(5): 2297-307] and that in the absence of β2-m, the MHC-I-peptide complex can not be formed [shields, 1998 (Supra)]. Moreover, it is known that at high temperatures (e.g., higher than 25 °C), the dissociation of the β2-m from the MHC-I molecule is faster, hence rendering instability to the MHC class I complex. Thus, the significant stabilization of the MHC class I complex of the present invention which is absent from the MHC-II complex prepared by Altman et al. results from the covalent linkage between the HLA and β2-m molecules which are expressed from a single polynucleotide.

In addition, in sharp contrast to the present invention which uses *E. coli* as host cells for expressing the MHC-I complex, Matsumura et al. used eukaryotic cells. In the eukaryotic system, the endogenously degraded peptides as well as the endogenously expressed HLA and β2-m polypeptides can form a complex and thus can be presented and detected on the cell surface similarly to the exogenously expressed HLA and β2-m polypeptides and the added peptide. Thus, using eukaryotic cells as an expression system, there is no way of discriminating the endogenous polypeptides and peptides from the exogenously expressed polypeptides (*i.e.*, via the expression vector) and the exogenously added peptides. In addition, following their purification, HLA-peptide complexes generated in eukaryotic cells should be stripped from the endogenous derived peptides to enable the binding of exogenous peptides of interest. However, the stripping process which utilizes acid [Rotzschke O, et al.,

1990, Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells, Nature. 348(6298): 252-4] results in destabilization of the MHC-I and β2-m complex [Rammensee HG, et al., 1993, MHC molecules as peptide receptors, Curr. Opin. Immunol. 5(1): 35-44; Urban RG, et al., 1997, The discovery and use of HLA-associated epitopes as drugs, Crit. Rev. Immunol. 17(5-6): 387-97]. In contrast, in a bacterial expression system such as that used by the present invention, the single-chain polypeptide is purified from the inclusion bodies as an insoluble protein and is subsequently refolded in a stable state in the presence of the peptide; as a result no peptide stripping and exchange is required.

In view of the claim amendments and accompanying arguments, Applicant believes that claims 5-15 are no longer rendered obvious by the prior art cited by the Examiner. Prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

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Enc:

Berko D, et al., 2005, Membrane-anchored beta 2-microglobulin stabilizes a highly receptive state of MHC class I molecules. J. Immunol. 174(4): 2116-23.

Shields MJ, et al., 1998, Characterization of the interactions between MHC class I subunits: a systematic approach for the engineering of higher affinity variants of beta 2-microglobulin. J. Immunol. 160(5): 2297-307.

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Rammensee HG, et al., 1993, MHC molecules as peptide receptors, Curr. Opin. Immunol. 5(1): 35-44.

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MHC molecules as peptide receptors

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The central unit for regulation of the specific immune system is a trimolecular complex made up of the T cell antigen receptor, the MHC molecule, and the MHC ligand. The third component is a peptide derived as a degradation product from a protein. During recent years there has been some progress in understanding the interaction between MHC molecules and their peptide ligands: MHC molecules are peptide receptors of peculiar specificity, being able to accomodate millions of different peptides provided they share some common features.

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Introduction

The function of MHC molecules is to collect peptides inside the cell and transport them to the cell surface, where they can be surveyed by T cells. The interaction between T-cell receptor (TCR), MHC molecule and the peptide presented by the MHC molecule is central to self/non-self discrimination, that is, to the main theme of the immune system. The purpose of this review is to discuss the relationship between MHC molecules and their natural peptide ligands, with special emphasis on MHC class I molecules, because more is known about these than class II molecules.

Two classes of MHC molecules

Most of the human MHC genes are encoded on chromosome 6; those of the mouse are on chromosome 17 [1]. Each species has dozens of MHC loci, some of which contain pseudogenes. Some of the MHC genes are extremely polymorphic. Klein [2] distinguished two classes of MHC genes, class I and class II, a distinction that well anticipated the different physiological functions of the respective gene products. MHC class I molecules are made up of a heavy chain, comprising about 350 amino acids, and a light chain, comprising about 100 amino acids. The latter is also known as β2-microglobulin. There are about 30–50 MHC class I loci in the mouse and at least 17 in the human, including pseudogenes [1,3]. Most of this review will deal with only five of them, that is, HLA-A and -B, and H-2K, -D, and -L. MHC class II molecules are made up of an α-chain, comprising about 200 amino acids, and a β-chain of about the same size. There are about six α loci and 10 β loci for class II genes in the human and at least four α and three β loci in the mouse [1,3]; this review will only deal with HLA DR, H-2A and H-2E molecules. Although MHC class I and class II molecules have many features in common, their function is quite different. Both classes will therefore be treated separately.

MHC class I molecules

Structure of class I molecules

The heavy chain consists of three extracellular domains, α1, α2, and α3, a transmembrane region [some non-classical class I molecules, however, are glycosyl phosphatidyl inositol (GPI)-anchored] and a cytoplasmic tail at the carboxyl terminus. The light chain, β2-microglobulin, is non-covalently attached to the heavy chain. Both α1 and α2 domains form a peptide-accommodating groove, as revealed by X-ray crystallography of an A2 crystal by Bjorkman and colleagues in 1987 [4]. The groove is bordered by two α-helices; the floor is a β-pleated sheet. The area of the groove is about 1 × 2.5 nm. The peptide appears to be an integral part of the protein complex, since empty class I molecules are thought to be rather unstable [5–8]. The peptide, which is generally between eight and 11 amino acids long, is tightly mounted in the groove in an extended configuration and certainly not as an α-helix [9•,10•,11–15]. Both amino and carboxyl termini of the peptide are tightly bound via H-bonds by conserved residues. In addition, two side chains of residues at particular positions of the peptide, one at the carboxyl-terminus, the other elsewhere, depending on the MHC allele, are held by allele-specific pockets of the groove [9•,10•,11–18]. Monopeptidic crystals indicated that the spatial distance between the peptide's amino and carboxyl termini is constant, whereas the number of amino acids is not; the H-2K^b molecule, for example, can accomodate 8-mers in a stretched configuration, and also 9-mers with a kink in the middle [10•].

Abbreviations

HLA—human leukocyte-associated antigen; MHC—major histocompatibility complex; TCR—T-cell receptor; TFA—trifluoroacetic acid; VSV—vesicular stomatitis virus.

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The induction of kinks may be promoted by proline or glycine residues of the peptide [16]. For many class I molecules, such as H-2K^b, H-2K^d, and HLA-A2, the vast majority of natural peptide ligands are of uniform length (8-mers for H-2K^b and 9-mers for many others) [16]. For other class I molecules, such as HLA-A11 and HLA-A31, it seems that they can accommodate peptides comprising eight to 11 amino acid residues, all with a fixed carboxyl terminus (charged for HLA-A11) (K Falk, O Rötzschke, M Takiguchi *et al.*, unpublished data). It seems, therefore, that the peptide backbone can bend more or less, to accomodate a different number of amino acids between the fixed MHC sites binding peptide amino and carboxyl termini as recently visulalized by X-ray crystallography of Aw68 molecules and associated peptides [19].

Technical approaches for isolating natural MHC class I ligands

Peptides can be dissociated from class I molecules by treatment with acid [trifluoroacetic acid (TFA), or acetic acid] [16,20–23,24•,25–27]. Peptide loaded class I molecules need not be purified to undergo such treatment; treatment of whole cell lysate with TFA brings the peptides into solution, which can then be detected by the respective T cells [20]. This was the approach used for the first isolation of minor histocompatibility antigens as well as virus-derived peptides from cells [20–22]. Acid extraction of whole cells has the advantage of yielding not only MHC-bound peptides but also other peptides, for example potential intermediates of the processing pathway [21,22,24•,25]. The disadvantages are that one needs specific T cells to detect the peptides of interest, and that the resulting material is generally too complex for sequencing (although there is one exception [25]). If the MHC molecules are first purified and then acid extraction of peptides applied, the resulting peptide mixtures are much purer and they lend themselves more readily to further isolation and sequence analysis [16,23,28,29••,30•,31•,32].

Allele-specific peptide motifs

One of the first natural class I ligands to be identified was Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val from influenza nucleoprotein [22,33]. This peptide is presented by H-2K^d molecules of infected cells. The tyrosine residue of several synthetic peptides is known to be important for binding to H-2K^d, as well as an alanine or leucine about eight or nine residues from the tyrosine [34,35]. Comparison of Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val with the H-2K^d-binding synthetic peptides (all being longer than nine amino acids) suggested that all natural H-2K^d ligands might be nonamers with a tyrosine residue at position 2 and some aliphatic residue at position 9 [33]. We considered then that an obvious experiment would be to sequence all the H-2K^d ligands as a mixture to see whether the hypothesis was correct [16]. Indeed, pool sequencing of H-2K^d-eluted peptides gave a strong tyrosine signal at position 2, isoleucine and leucine at position 9, and lack of significant signals at position 10. Pool sequencing of peptides eluted from other MHC class I molecules indicated that each MHC allelic product has its own individual rules for peptide ligands, in most cases with determined peptide length and with two positions within the peptide of conserved occupancy, called anchor positions. The information obtained in this way is summarized as peptide motif [16]. Such motifs can also be determined by comparing many individual peptide ligands [28]. In the case of H-2K^d, a motif based on binding of synthetic peptides was found to be essentially identical to the basic motif based on natural ligands [36]. The basic motifs of some class I alleles are shown in Table 1. In addition to the basic motif information, i.e. allele-specific length, anchor position and occupancy, the different motifs have more subtle characteristics, such as preferential use of certain residues at certain positions; for example, HLA-A2 ligands have a preference for valine, isoleucine, leucine or alanine at position 6, and H-2K^b ligands prefer tyrosine at position 3 [16]. Such positions have been called auxiliary anchors. If one attempts to use the allele-specific peptide motifs for the prediction of natural T cell epitopes [37], as has already been done successfully [38•,39•], one should consider the more subtle preferences as well.

Table 1. Natural MHC ligands. Examples of basic allele-specific peptide motifs for class I molecules.

Class I allele	Position									Reference
	1	2	3	4	5	6	7	8	9	
H-2K^d	X	Y	X	X	X	X	X	X	L I	[16]
H-2K^b	X	X	X	X	F Y	X	X	M I L		[16]
H-2D^b	X	X	X	X	N	X	X	X	I L M	[16]
HLA-A2	X	L	X	X	X	X	X	X	V	[16,29••]
HLA-B27	X	R	X	X	X	X	X	X	X*	[28]

The common features of peptide ligands of the individual MHC molecules are shown. X stands for any residue. Amino acids are represented by the one letter code in bold. The side chains of these amino acids contact the allele-specific pockets in the class I molecule. For example, K^d-ligands are nonamers, with anchors at positions 2 (mainly for the aromatic residue Tyr) and 9 (for the aliphatic residues Leu and Ile). The more detailed characteristics of the individual motifs have been omitted for clarity.
* Predominant usage of Arg or Lys

The side chains of anchor amino acids as well as of auxiliary anchors are thought to be held by the allele-specific pockets of the MHC class I peptide binding groove. The remaining residues of the peptide ('point ing up') should then be available for contact with the TCR [9•,10•,11–18,40–46].

Natural MHC class I ligands

The first three natural MHC class I ligands were identified in 1990 [22,23]. All were of viral origin, and the approach used was to compare in T-cell assays the natural MHC ligand with synthetic peptides; in the case of the H-2K^b-restricted vesicular stomatitis virus (VSV) peptide Arg-Gly-Tyr-Val-Tyr-Gln-Gly-Leu, the natural ligand, isolated by high performance liquid chromatography, was partially sequenced. In these instances, knowledge of the viral protein sequence from which the peptides were derived was mandatory for identification. These and similar experiments also showed that exact peptide length (of synthetic peptides to be tested) is important for optimal T-cell recognition, since adding or removing one residue from the natural peptide size can decrease T-cell recognition (and MHC-binding) by several logs [33,34,47–49].

In 1991 the first natural MHC ligand was sequenced directly in the absence of information on the protein from which it originated [16]. It was found later that this peptide, the H-2K^d-ligand Ser-Tyr-Phe-Pro-Glu-Ile-Thr-His-Ile, is derived from the protein tyrosine kinase JAK1 [50] (A Wilks *et al.*, unpublished data). Since then, the number of natural MHC ligands that have been characterized is growing exponentially [24•,25–28,29••,30•,31•,39•,51•,52,53]. Most ligands known to date are self peptides, i.e. peptides derived from normal cellular proteins, representing the physiological MHC class I ligands. These come from all kinds of proteins synthesized in the cell and include proteins like histones, heat shock proteins, enzymes and so on. Relatively few natural class I ligands derived from foreign proteins, e.g. from pathogens, have been identified since 1990 [39•,51•], although the allele specific peptide motifs combined with T-cell recognition data of synthetic peptides, and sometimes peptide binding experiments, allowed the prediction of a large number of candidates for natural ligands (reviewed in [52]). Some examples of endogenous, as well as foreign peptides, naturally found associated with class I (or class II) molecules are listed in Table 2.

A technical development greatly improving direct sequencing of MHC eluted peptides was the use of tandem mass spectroscopy [29••]. This technique is ideally suited to dissect the thousands of different peptides eluted from a given MHC molecule, to obtain sequence information on individual peptides of relatively high abundance and to roughly estimate peptide complexity. Still a technical challenge, however, is to directly identify the peptide antigen recognized by a given T cell, if the origin of the peptide is unknown, as is the case, for example, for minor histocompatibility or tumor-associated antigens (the former are peptides derived from normal self proteins that can be recognized by foreign T cells). One of the reasons is the relatively low abundance (100–400 copies per cell) of most natural class I ligands as recognized by T cells of immunological interest [33,38•,51•,53].

Processing of MHC class I ligands

It is not known where and how MHC class I ligands are processed. Most peptides appear to be degradation products of proteins, although some alternatives have been discussed [54–56]. Many proteins donating peptides to class I molecules are located in the cytosol, and proteins artificially loaded into the cytosol can yield class I ligands [57–59]. In addition, however, probably all proteins synthesized in a cell, including those in the mitochondria [60,61], can supply class I ligands. The following is a widely held view for class I restricted processing: proteasomes cut cytosolic proteins into peptides, and products of *Tap1* and *Tap2* genes transport these peptides across the endoplasmic reticulum membrane where they bind to MHC class I molecules. This model, however, is still speculative regarding the postulated function of TAP molecules [62–64] and fails to take into account the observed MHC-dependency of peptide occurrence in cells [21,22,24•,51•,65,66•,67,68]. (For example, the male specific H-Y peptide, recognized by H-2D^b restricted T cells, is apparently absent from male cells not expressing H-2D^b) [21]. With regard to this aspect, and on account of the structural features of the consensus motifs, we

Table 2. Natural MHC ligands. Examples of natural ligands of MHC class I and class II molecules

MHC allele	Peptides*																							Origin	References
H-2K^d									S	Y	I	P	I	I	T	H	I							Protein tyrosine kinase JAK1	[17,50]
H-2K^b									R	G	Y	V	Y	Q	G	L								VSV nucleoprotein	[22]
									S	I	I	N	F	E	K	L								Ovalbumin	[30•]
HLA-A2									T	L	W	V	D	P	Y	E	V							Unknown	[29••]
HLA-B27									R	R	Y	Q	K	S	E	F	I							Histone H3	[28]
									R	R	I	K	E	I	V	K	K							HSP 89a	[28]
H-2D^b	S	P	S	Y	V	Y	H	Q	F	E	R	R	A	K	Y	K								MuLV env. protein	[86•]
	S	P	S	Y	V	Y	H	Q	F	E	R	K	A	K										MuLV env. protein	[86•]
H-2A^d	E	E	Q	F	Q	Q	I	R	L	Q	A	E	I	F	Q	A	R								Mouse apolipoprotein E	[88•]
			Q	F	Q	Q	I	R	L	Q	A	E	I	F	Q	A	R								Mouse apolipoprotein E	[88•]
H-2A^k	D	G	S	T	D	Y	G	I	L	Q	I	N	S	R											Hen egg white lysozyme	[92]
					D	Y	G	I	L	Q	I	N	S	R	W	W									Hen egg white lysozyme	[92]
HLA-DR1	L	R	K	P	P	K	P	V	S	K	M	R	M	A	F	P	E	E	N	Q	A	K	P	M G	Invariant chain	[90]
										K	M	R	M	A	F	P	E	E	N	Q	A	K	P		Invariant chain	[90]
					A	F	E	F	F	R	A	N	A	Q	F	S	R	K	F	D					Unknown	[89]

* Anchor residues in bold. Amino acids are represented by the one letter code. HSP, heat shock protein; VSV, vesicular stomatitis virus

speculated that the MHC itself might have an instructive role in peptide processing, in that larger peptide precursors, cut by conserved endopeptidases (proteasomes?), would first bind to MHC molecules and then be trimmed to the final size specific for the particular MHC allele [16,22]. Trimming of a leader peptide, probably in the endoplasmic reticulum, is suggested by the presence of longer peptides in addition to the core nonamer bound to HLA-A2-molecules [29••,30•,31•]. Our speculative model would also explain the fact that in all human tissues and mouse strains examined, cells are able to process the very same peptides for one particular class I molecule, H-2K^b [21,24•,65,66•,67,68]. If one assumes, according to the other, more popular, model that the final peptide is produced before its first contact with the MHC molecule, all cells in a white mouse, for example, should constantly produce all the tens of thousands of MHC class I ligands not only for all the black, brown and other members of the species but also for all other mammals [69]. In addition, this high number of potential ligands should have an extremely short half life, since so far they have not been detected, in spite of thorough experimental attempts [66•].

The pathway of class I restricted processing is certainly an area attracting much attention at present. Particularly interesting molecules are the *Tap1* and *Tap2* gene products, proteasomes, and other potential transacting elements [70]. TAPs are essential for proper antigen processing; their exact function, however, is not known. It is known that class I molecules are peptide transporters (from the endoplasmic reticulum to the cell surface) but it is not known whether TAPs also transport peptides.

Considerations on the role of MHC class I molecules

Peptide selection on the cell surface of peptides derived from cellular proteins provides the immune system with the means to control the cell's interior. For a given individual, this control covers only a small portion of the expressed genome. Each individual in an outbred population (man or mouse) expresses four to six different class I molecules. Each molecule species can present an estimated 1 000 different peptides on a cell [29•,33,69]; since the peptide specificities are different, depending on the class I allele, each molecule species presents a different set of peptides. The peptide selectivity of a given class I molecule is such that about 1 per cent of random (nona-) peptides fit to it [33]. Thus, only about 4–6 per cent of the expressed genomic sequences fit into class I molecules in an individual but due to MHC polymorphism, a much higher percentage of these sequences fit to the various class I molecules expressed in an entire species. During their ontogeny, T cells are made tolerant [71,72] to those self peptides that are actually presented by MHC molecules, but T cells are not tolerant to the remaining 95 per cent of self protein sequences [68,73,74]. Although not sufficient to control for every point mutation in the expressed genome, this system is sufficient to report the occurrence of new (non-self) gene products on the cell surface, since about one peptide (1 per cent) fitting into a particular class I molecule should be present within a protein sequence of 100 amino acids. Thus, T cells have a chance to detect cells expressing genes from invading viruses or other organisms with cytosolic parasitism. In addition, the system should allow the detection of proteins not usually expressed (and, therefore, not inducing tolerance) such as genes normally involved in embryonic development that are aberrantly expressed in malignant cells [75–79]. Normal proteins expressed at unusual abundancy might also be detectable by T cells, since they can detect quantitative differences in the peptide copy number presented by cells [51•]. Thus, the system of class I restricted peptide presentation and class I restricted T cells is able to efficiently control intracellular viral and certain other (cytosolic) parasites [39•,80–82], and should also have some control on malignancies [75–79].

Could the system be more efficient if the cell would present only the foreign peptides? Yes, indeed, but the cell cannot distinguish self from non-self; the immune system had to evolve a sophisticated system for this challenge, consisting of many cells, tissues, and organs, most notably the thymus and lymphocytes.

Class II

Structure of class II

It is believed that class II α and β domains form a groove that is similar to that formed by the the α1 and α2 domains of class I molecules [83,84]. This notion has been derived from modeling the class II sequence according to class I crystallographic data [4], since a photograph of a class II crystal has been taken [85•] but no X-ray crystallography has been reported yet. From the nature of natural peptide ligands of class II molecules reported since 1991 [86•,87,88•,89–92], it can be deduced that there must be certain differences in the peptide holding groove compared with that of class I molecules. The most notable difference is that the ends of the groove appear to be open, that is, to allow overhanging of both amino and carboxyl termini of bound peptides. The latter are probably in an extended conformation rather than in the form of α-helices.

Natural ligands of class II molecules

Our knowledge of class II physiology was more advanced than that of class I physiology for many years. T helper cells were first found to be MHC class II restricted in their interactions with B cells two years before MHC class I restriction of cytotoxic T cells was demonstrated in 1974. The first naturally processed MHC ligands were also eluted from class II molecules, although they were not identified [93]. Our understanding of class I molecules surpassed that of class II with the X-ray crystallographic study of class I structure in 1987 [4] and with the identification of natural ligands in 1990 [22,23].

The first natural MHC class II ligands to be identified were eluted from mouse H-2A^b and H-2E^b molecules [85•]. These peptides were between 12 and 18 amino acids

long; the carboxyl terminus appeared to be 'ragged', i.e. peptides with a given core sequence could be extended at the carboxyl terminus to give 15-, 16-, 17-, or 18-mers. Again, the use of tandem mass spectrometry provided a boost for the identification of naturally processed class II ligands [88•]. Use of this technique allowed the detection of up to 2000 different ligands on a single class II species, H-2A^d, although only the 12 most abundant ones have been sequenced. To date, information is published on H-2A^b, -E^b, -A^d, -A^s, -A^k, and HLA-DR1 peptide ligands, representing some 40 natural peptides, some of which are indicated in Table 1 [86•,87,88•,89–92].

Both the amino and carboxyl termini are ragged; their length can vary between 12 and 25 residues, with the majority apparently around 15 amino acids. For T-cell recognition, the length of the peptide seems to matter little, as long as a certain core is maintained [91,92].

Allele-specific peptide motifs?

The sequencing of the first 13 natural MHC class II ligands (from H-2A^b and H-2E^b molecules) did not indicate any obvious motifs [86•]. Subsequent studies on the 11 most abundant H-2A^d associated peptides indicated that peptide binding motifs established earlier using synthetic peptides [94,95] were contained within the natural ligands that associate with H-2A^d [88•]. Similarly, HLA-DR1 eluted peptides were aligned to conform to a motif of three anchor-like positions [90]; this motif, however, did not exactly conform to HLA-DR binding motifs established with synthetic peptides [89,96–102]. Common to all studies analyzing natural class II ligands is the ragged end of peptides; that is, peptides containing a given core sequence were found in various lengths, with extensions both at the amino and carboxyl termini. Thus, there appear to be allele-specific peptide motifs in class II ligands, although the motifs are not as obvious as with class I ligands, and the anchor-like positions may allow a broader spectrum than in the case of class I molecules. Although crystallography studies on class II molecules have not been reported yet, the information on class II restricted peptides suggests that allele-specific pockets will also be found in the groove, and that both ends of the groove are 'open' to allow overhanging of peptides at both sides, explaining the different length of peptide ligands. This is illustrated in Fig. 1.

Most class II ligands are of endogenous origin

It is a widely held misconcept that class II molecules predominantly present peptides from exogenous anti-

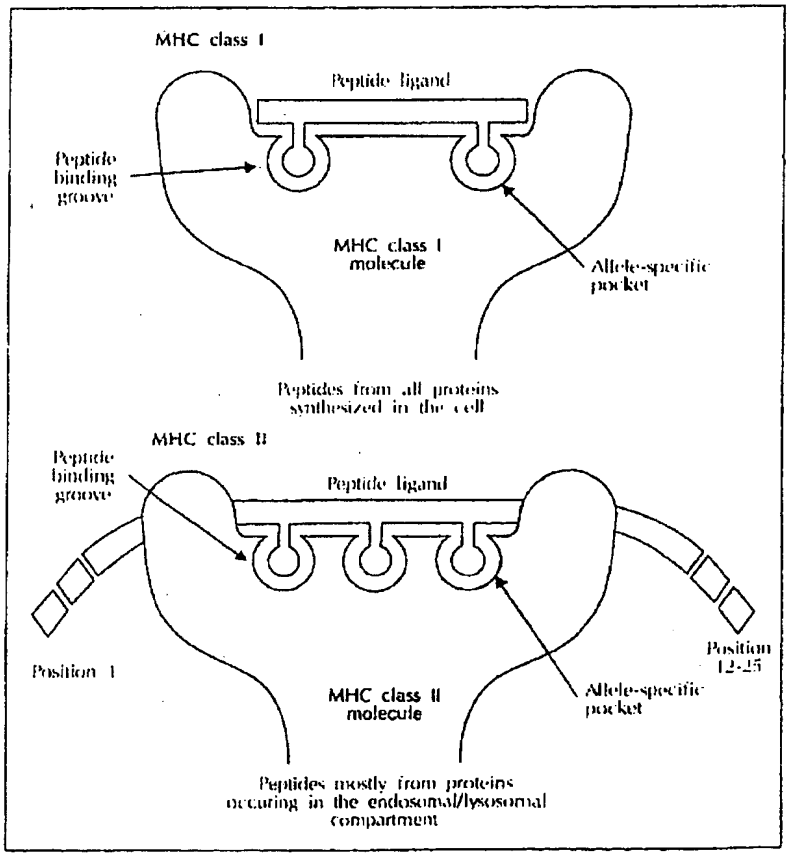

Fig. 1. A simplified view of MHC class I and II molecules as peptide receptors. Class I molecules hold peptides of eight to 11 amino acids with both amino and carboxyl termini tightly fixed in the groove. Two side chains of anchor residues of the peptide are protruding into complementary allele-specific pockets of the groove. Class II ligands, consisting of 12–25 residues, are probably also held in the groove by anchoring two or three peptide side chains into complementary structures of the class II groove. In contrast to class I, however, both peptide ends are not fixed in the groove but are allowed to 'hang out' of the end of the groove.

gens, i.e. from antigens taken up by the antigen-presenting cell. As can be seen from the list of known natural class II ligands [86•,87,88•,89–92], most are actually endogenous to the cell. Peptides are derived from other MHC or MHC-associated molecules, e.g. HLA-A2, H-2A or -E, from the invariant chain, retroviral protein, or the transferrin receptor. In addition to these endogenous peptides, foreign peptides are also found, e.g. peptides derived from bovine serum albumin present in the cul ture medium used to propagate the cell line [86], or peptides derived from antigen deliberately 'fed' to the cells in high concentration, such as hen egg white lysozyme [92]. Common to most of the proteins donating peptides to class II molecules is their potential to occur in the endosomal/lysosomal compartment of the cell, no matter whether this is achieved by phagocytosis of exogenous proteins, or by directing endogenous molecules into this compartment.

Thus, class II molecules predominantly present peptides derived from proteins occurring in, or directed to, the endosomal/lysosomal compartment of the cell, whereas MHC class I molecules can present peptides derived from all the proteins synthesized in a cell.

How do the cells process the peptides?

MHC class II ligands are probably produced by lysosomal enzymes (reviewed in [103]). Several enzymes, among them cathepsin D, are involved, as indicated by the different sensitivity of particular ligands to protease inhibitors (reviewed in [104]). It is not known, however, whether the lysosomal enzymes produce the final size ligands before binding to class II molecules, or whether the ligand is trimmed after binding, with protection of the peptide core sitting in the groove ('determinant protection') [105]. The ragged ends of class II ligands may suggest limited action of exopeptidases on the ligands after binding.

Why are MHC class II molecules not flooded with ligands in the endoplasmic reticulum, where they are assembled, as are class I molecules? One reason for this is the blocking of the peptide binding site by the invariant chain. Only after removal of the latter in the endosomal/lysosomal compartment do the peptides have access to the class II cleft [106].

T cell function and MHC class II molecules

The function of class II restricted $CD4^+$ T cells is twofold: firstly, they 'help' other antigen-specific lymphocytes (B cells and other T cells) to differentiate and to become activated; and secondly they attack foreign antigen presented by MHC class II positive cells, either directly or by activation of non-specific cells like macrophages or granulocytes. Both functions are mediated and regulated by differential cytokine production [107]. For the interaction between T helper cells and B cells, the requirement for the T cells to recognize the class II ligand on B cells forces close contact between the T cell and the B cell recognizing the same antigen and allows directed delivery of cytokines. The epitopes recognized by the TCR, however, are usually not the same as the antibody epitopes.

In addition to positive immune responses, recognition by T cells of peptide ligands associated with MHC molecules may also lead to tolerance, not only in the thymus or during T-cell ontogeny. Resting B cells, for example, presenting antigen acquired by receptor-mediated phagocytosis, have been hypothesized to induce anergy in mature T cells that recognize them [108,109]. This could be a mechanism for maintaining self tolerance in the mature T-cell compartment; the mechanism might also be used to induce peptide-specific tolerance in T cells artificially.

Concluding remarks

Comparing the peptide receptor characteristics of MHC class I versus MHC class II molecules, two major differences are evident (see also Fig. 1). First, the majority of peptide ligands of most class I molecules have a distinct length (eight or nine residues), depending on the allele, whereas MHC class II ligands may vary considerably, from 12–25 residues. The second difference is regarding the peptide motifs. Class I molecules have distinguished allele-specific motifs which become obvious on aligning as few as nine or 10 natural ligands, and are easily accessible by pool sequencing of the total ligand mixture. Peptide motifs of class II molecules are not as obvious, especially if one looks at a few ligands only. Nevertheless, class II specific peptide motifs appear to exist, and additional work is required to establish clear motifs for the different class II alleles.

Knowledge of such motifs, for class I and class II molecules, is useful to predict natural T-cell epitopes. Other subjects of current and future interest include: firstly, the peptide specificities of HLA-C [110] and of non-classical class I molecules (in addition to the Qa-2 motif [111] and the information on H-2M3, which is specific for N-formylated peptides [60,61]), and also peptide specificity of HLA-DQ and HLA-DP molecules; secondly, the fine dissection of the molecular interactions in the peptide–MHC–TCR relation (in particular, which atoms of the three molecules contact each other?); thirdly, the dissection of the processing pathways involved in processing of MHC ligands; and finally, the identification of peptides expressed on thymic epithelium and elucidation of their role in positive selection.

The detailed information of the peptide receptor function of MHC molecules, especially the structural features of their ligands, should be useful for applied immunology, including vaccine development, immunotherapy of malignant and infectious diseases, and prevention and therapy of autoimmune diseases.

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Due to constraints on the space allowed for this review much of the work that led to the model of peptide processing for MHC class I molecules has not been cited.

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The Discovery and Use of HLA-Associated Epitopes as Drugs

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ABSTRACT: MHC receptors "display" peptide fragments to T cells. These peptides are predominantly derived from proteins expressed within or ingested by the presenting cell. Since empty MHC molecules are highly unstable, peptide ligands are bound prior to MHC surface expression and the ensuing $t_{1/2}$ off rates are often on the order of days. It is the remarkable stability of MHC/peptide complexes, which provide us an opportunity to purify MHC molecules from infected, transfected, or antigen pulsed cells and subsequently identify the naturally processed peptides being presented. On the other hand, the stability of MHC/peptide complexes substantially reduces the potency of parenterally administered peptides *in vivo*. Using serial immuno-affinity chromatography and mass spectrometry, naturally processed peptides can be identified. When these peptides are then encoded into nucleic acid and delivered parenterally, they are highly immunogenic. Application of these techniques to induce vigorous CTL responses will be discussed.

KEY WORDS: peptides, antigens, vaccines, DNA, microsphere, cytotoxic lymphocyte.

I. THE NATURE OF HLA-PEPTIDE INTERACTIONS

Proteins encoded within the class I and class II regions of the major histocompatibility complex participate in immune recognition of pathogens by binding and transporting to the cell surface small immunogenic peptides.[1] Class I and class II genes are the most polymorphic gene families in the human genome. This led to the proposal that a significant survival advantage has been afforded by increasing the diversity and number of these receptors. This concept is also supported by the binding mechanics of these molecules. Unlike most receptor-ligand interactions where specificity is imperative, HLA molecules strive to accommodate as many different ligands as possible. Most often the peptides presented by HLA molecules are remnants of full-length proteins that have undergone partial proteolytic degradation.[2,3] As essentially random combinations of twenty different amino acids, these peptides represent a remarkably diverse collection of chemical entities or ligands. From a receptor/ligand point of view, it is difficult to imagine how a single receptor could bind to all these peptides and do so with high affinity when these ligands are so seemingly different. The answer to this puzzle is that HLA molecules do not focus their attention on the differences between peptides but rather on the features that all peptides have in common. The universal features that HLA molecules have evolved to recognize are the peptide bonds between each amino acid and in the case of class I receptors to the NH_2- and -COOH termini of peptides.[4,5] HLA molecules can bind peptides with low nanomolar affinities by employing numerous cooperative interactions, which together add up to the overall high affinity.

Although a given HLA molecule can bind a large number of different peptides, it cannot bind all peptides. This fact resulted in the selective pressure to increase the number of receptors expressed. But, if HLA molecules use common structural motifs to bind to peptides why are they incapable of binding all peptides? Most peptides

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in the size range bound by HLA molecules are too small to maintain any rigid tertiary structure. Our working model of the HLA-peptide interaction suggests that during the binding process, an initial interaction at only a few contacts captures the ligand while the non-bound portion is allowed to freely rotate at each peptide bond in an attempt to increase the number of contacts. This model is supported by observations of weak initial affinities, which then convert to higher affinity-stable complexes.[6,7] The end-state conformation minimizes potential steric hindrances and appropriately aligns the electrostatic interactions of each charged amino side chain with those elements present within the receptor's ligand binding groove. If during this process irreconcilable conflicts are encountered, the weak preliminary interactions, which initiated the binding process, will prove to be insufficient to maintain a stable conformation and the ligand will be released.

Notwithstanding the above, the atomic idiosyncrasies (derived from polymorphisms with the binding pocket) of HLA molecules will tend to favor certain types of amino acid side chains at particular positions of the ligand. Although these amino acids only moderately participate in the total binding energies between receptor and ligand, they may make significant contributions during the critical early interactions between HLA and ligand. Small contributions in binding energy during this time period will dramatically increase the likelihood that the remaining cooperative sets of interactions proceed.

Interestingly, class I and class II HLA molecules are incomplete with respect to the structural requirements needed to completely fold. Obligate chemical contacts are contained within the bound peptide ligand and as a result "empty" class I and class II HLA molecules are unstable and unfold rapidly. This is not the case for their close structural relative, the neonatal Fc Receptor.[8] This implies that acquiring the appropriate ligand before achieving the tertiary fold is important. When one considers the fundamental role class I and class II molecules play as the first step in the immune recognition event, tight control over ligand acquisition makes sense.

HLA-peptide complexes have significantly slower offrates than most structurally rigid receptors-ligand systems. The offrates for most HLA-peptide complexes are similar in duration to the half-life of nascent HLA molecules. From a practical standpoint these facts have significant implications. Because HLA molecules and their ligands are assembled within intracellular vesicular compartments and the $t_{1/2}$ are often on the order of days, sparingly few HLA complexes on the cell surface are "empty" or "open" for binding of exogenously encountered peptides. Thus, a peptide-based pharmacologic agent, when administered parenterally, has little chance of loading HLA molecules (further discussed below) and remains immunologically inactive outside the context of HLA presentation. Yet, because of these slow offrates, HLA molecules can be purified from cells, and an analysis can be performed on the repertoire of peptides being presented.

II. DISCOVERY OF IMMUNOLOGICALLY ACTIVE PEPTIDES

One approach for identifying minimal epitopes has been to synthesize partially overlapping peptides covering the entire amino acid sequence of the target protein and then screen each of these peptides for their ability to bind to HLA molecules or elicit an immune response. The primary obstacle in applying this technique is that a significant fraction of the high-affinity-binding synthetic peptides identified do not correspond to the naturally processed epitopes presented by the cell and thus are artifacts. A more practical obstacle is the expense of synthesizing overlapping peptides covering the entire length of the protein, especially in those instances where the target protein is of an extended length. Nevertheless, several immunogenic peptides were identified using this approach.[9-12]

As an alternative to screening peptide libraries, techniques have been developed to identify naturally processed peptides from the surface of cells grown in tissue culture or tumors removed from patients. The pioneering work of Buus et al. was the first to show that acid treatment of HLA molecules released low-molecular weight proteinaceous material.[13] Refinement of this technique applied to purified HLA-peptide complexes has resulted in a substantial amount of sequence information defining HLA-associated peptides.[2,14-25]

The majority of these applications utilize immunoaffinity chromatography to purify the HLA molecules from either cell lines or human tissue samples.[26] Originally, soft carbohydrate gels were used for the preparation of chromatographic packings because they were inexpensive, easily derivatized, of high porosity, and useful for laboratory scale preparative separations. In our laboratory this protocol was modernized to improve the protein yield, reduce the number of manipulations, and eliminate the exposure of HLA-peptide complexes to extensive dialysis.[27] By automating the purification system, the time required to obtain highly purified HLA-peptide complexes can be reduced from several days to a matter of hours. This reduction in time is important to maintain the integrity of the HLA-bound peptide repertoire. Although most of these complexes are quite stable, the receptor-ligand interaction is not covalent and peptides are continually being released over time, as described in the previous section. Hence, lower affinity and lower occupancy peptides are naturally more difficult to isolate and analyze. In some instances immunogenic peptides fall into this category.[22,28] Thus, by implementing a faster purification scheme, a more complete analysis of the entire bound peptide repertoire is achieved, leading to a better chance of isolating and identifying relevant immunogenic epitopes. However, speed is not the only advantage of an automated system. Along with the advantage of faster overall purification times come improvements in reproducibility afforded by this approach that are necessary to increase the sensitivity of this technology for the analysis of complex biological samples.

The automated system described above consists of tandem HPLC columns linked in series to achieve immunoaffinity separations of several HLA molecules from a single sample. In this approach, mAbs are directionally attached to high-strength, large throughpore perfusion sorbents that allow fast velocity flowrates (up to 20 mL/min) and also facilitate the cleaning/recycling of columns after protein/lipid fouling.[29] This system was designed with multiple high-pressure switching valves, which allow appropriate flow paths for automated column loading and serial elution of up to five individual mAb-specific immunoaffinity columns (R. M. Chicz, unpublished results). These modifications empower a single system to automatically purify up to five allotype specific HLA molecules from a single lysate preparation without manipulation of the effluents or reloading of collected fractions. Because this is a modular system, additional high-pressure switching valves can be added to increase the number of individual columns to be eluted. This system is capable of both complex immunoaffinity protein purification as well as sensitive analytical reversed-phase chromatography (RPC) peptide separations, contiguously.[27] The effluent from the RPC column is split and single microliter aliquots robotically deposited onto a matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) target plate, while the remaining material is transferred into bioassay plates or storage vials. Next, an acidic matrix is applied directly on the sample plate and the peptide complex is crystallized. Once complete, the peptide samples are ready for immediate automated mass analysis.

The next step is to catalog the repertoire of peptides that are presented. To achieve this, a mass spectrometer capable of high sensitivity analysis is required as the monitoring device. Four prominent features make MALDI-TOF/MS an especially attractive analytical tool for this analysis. First, MALDI-TOF/MS spectra tend to be less complicated than those collected using electrospray ionization mass spectrometry (ESI/MS) because the ionization process favors the formation of single (1+) ions rather than multiply charged ions (1+, 2+, 3+, etc.). This is an important consideration when comparing spectra of complicated samples. Second, this technique uses minimal amounts of sample, sub-femtomole amounts for mass analyses and femtomolar amounts for sequence analyses. Third, the mass accuracy and superior mass resolution afforded using this technique are not achievable using alternative mass spectrometry analyses. Finally, primary sequence information can be generated using two complementary modes of daughter ion fragmentation. The first two considerations described above are self evident, but the remaining points are subtle and will be expanded on below.

A reflectron time-of-flight mass spectrometer is capable of collecting mass spectra in several modes of operation. Peptide fractions are first

screened for complexity and relative abundance using the linear mode of analysis, which has a lower resolving power and mass accuracy, but a higher transmission efficiency for complex mixtures. These spectra provide an accurate catalog of individual peptides present and consequently a precise time of elution from the RPC column. Because each fraction from the primary RPC separation can contain hundreds of individual peptides, high resolution combined with mass accuracy is the only method that can reliably screen the fractions for complete peptide characterization. Thus, the next analysis is performed in the reflector mode which increases the resolution and mass accuracy of the spectra by increasing the flight time of the ionized species. For example, techniques with lower resolving power (i.e. ion trap or triple quadruple mass spectrometers with normal resolution of ~1000 to 2000 in the m/z = 1000 to 2000 range at femtomole sensitivity) have difficulty characterizing peptides with mass differences of 1 to 3 Da or less (Figure 1a). The difficulty is mostly due to the inability of these alternative techniques to properly resolve the isotopic distribution of a single peptide. MALDI-TOF/MS instruments equipped with extended flight paths and delayed extraction ionization fields

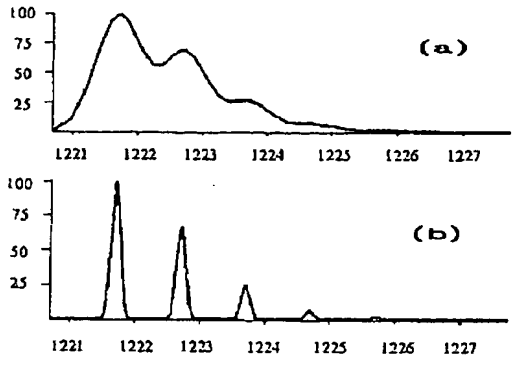

FIGURE 1. Isotopic resolution profiles for several different mass spectrometry techniques. The upper panel represents the theoretical isotopic resolution for a m/z = 1221.7 with a resolution of 1500. This is a typical value for electrospray ionization triple quadrupole mass spectrometers under limiting sample conditions. The lower panel represents the actual mass spectra collected in the high resolution reflector mode for the same peptide using MALDI-TOF/MS.

can achieve superior mass accuracy and resolution (~15,000) (Figure 1b),[30,31] at the femtomole and even attamole level. The exceptional performance of this instrumentation enables the reliable collection of multi-component spectra while permitting the mathematical subtraction of one spectra from another. Coupled with highly reproducible chromatographic separations, subtractive analysis of naturally processed peptides from antigen pulsed and non-pulsed cell lines can be performed. The application of this technology is utilized to identify novel HLA-associated peptides derived from immunogenic target proteins without the aid of T cell assays.

Another advantage of MALDI-TOF/MS relates to its ability to generate sequence information on peptide samples. Fragment ions can be generated in reflectron MALDI-TOF/MS by a phenomenon described as post-source decay (PSD).[32] Briefly, the sample analyte ions undergo "delayed" fragmentation/neutralization reactions during flight stemming from multiple collisions with matrix molecules during gas phase plume expansion and ion acceleration. It appears that MALDI-TOF/MS is unique in forming pre-excited precursor ions that move at a fairly high kinetic energy over a long distance where they can undergo uni-molecular decomposition with or without further collisional activation.[33] Using PSD analysis, complete sequence information can be generated from the daughter ion fragmentation patterns (Figure 2). The fragmentation patterns are different from those observed using high energy 4-sector instruments or other tandem mass spectrometers, such as electrospray triple quadrupole instruments. Furthermore, sensitivity is at least two orders of magnitude better than the aforementioned mass spectrometry approaches due to higher overall yield of fragment ions and higher ion transmission inherent in TOF instruments.[33] However, to enhance PSD analysis even further, a collision cell can be introduced to the system. With a collision cell in place, high energy collision-induced dissociation (CID) spectra can be collected, which produce complementary fragmentation patterns as compared to PSD spectra. The combined data sets produce additional structural information for the sequence determination of unknown peptides (Figure 3). Unfortunately, there are some practical limitations inherent with PSD

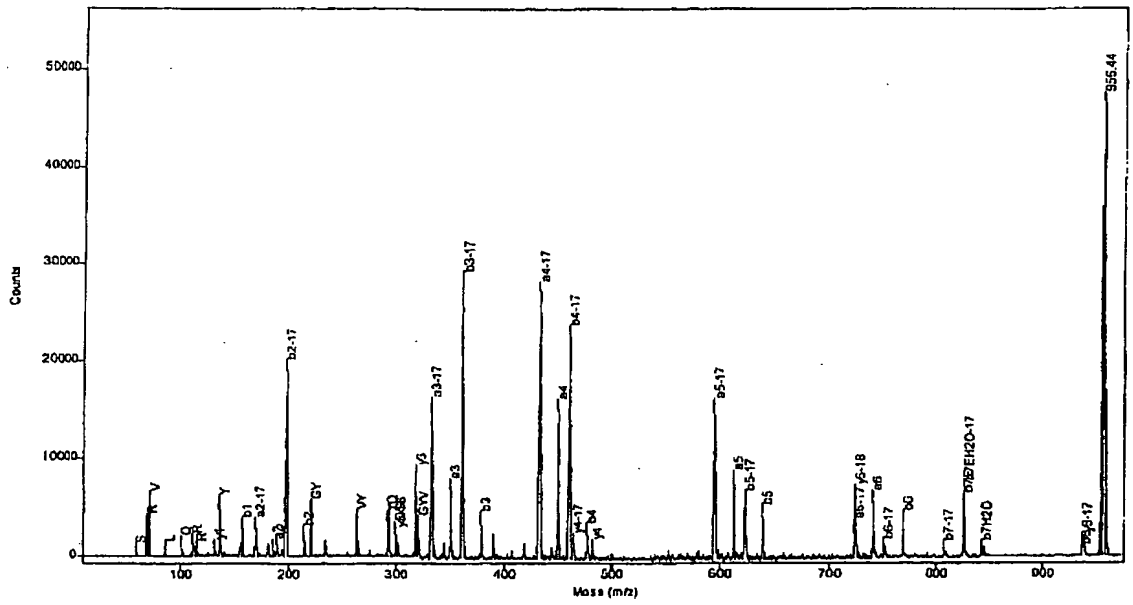

FIGURE 2. PSD fragmentation pattern from MALDI-TOF/MS analysis of a MHC-associated immunogenic peptide from vesticular stomatitis virus. A complete daughter ion fragmentation pattern is observed.

analyses. The current instrument design of commercial MALDI-TOF mass spectrometers is optimized for high resolution data collection, not sequence analysis. This characteristic of the instrument design is most evident when analyzing complex multi-component mixtures. Although the linear and reflector mode are capable of detecting most of the individual ions in a complex mixture, the suppression effects resulting from the plume ionization of a multi-component mixture are manifested by a decrease in sensitivity during PSD analysis. Innovations in ion gating and detector design are addressing this issue that should result in an increase in sensitivity for sequence analysis (Proceedings of the 45th ASMS Conference on Mass Spectrometry and Allied Topics, Palm Springs, CA, 1997). Unfortunately, the rapid progress of this technology still needs to await applications testing and marketing before commercial instruments are available.

A complementary technique to MALDI-TOF/MS for the sequence analysis of low femtomole amounts of peptide is ion-trap mass spectrometry. Ion-trap mass spectrometry was recognized as an emerging technology for the sequence analysis of HLA-associated peptides prior to the availability of commercial instruments.[27] In the interim, improved mass accuracy, resolution and sequencing capabilities were achieved on commercially available MALDI-TOF mass spectrometers. However, the combination of these two technologies may present the optimal manner by which peptide sequence identification can be efficiently obtained from complex samples. The rationale for this proposal is listed below. First, the mass range of ion-trap instruments has recently been extended to include linear mass calibration and ion fragmentation for peptides.[34,35] With these advances in place, several commercial ion-trap instruments are now available. Briefly, the strength of the ion-trap technology is the capability to isolate a given ion while ejecting all the nonselected ions from the instrument, hence the name ion-trap. This is accomplished through the use of non-linear multipole fields, advanced resonance frequency electronics, and optimized ring and endcap designs in the trap, which enhance the ion ejection speed and extend the useful mass range of the instrument. The end product is the ability to perform multiple fragmentation experiments on a given ion (known

as $MS^{(n)}$), which extends the amount of information collected from peptide sequencing. This is manifested by performing a ZoomScan or limited mass range scan on a known mass. In this mode, the instrument can operate at high sensitivity and resolution, but at the cost of scanning only a limited mass range. The price for this optimization is the inherent weakness of lower sensitivity and resolution of normal full scan spectra of the parent ions. The decreased sensitivity and resolution compromises the detection of most ions in complex mixtures. For these reasons, the combination of MALDI-TOF/MS with ion-trap MS may lead to faster sequence identification of HLA-associated peptides.

Mass spectra collected using reflector MALDI-TOF/MS analysis normally have a mass accuracy near 0.01%. This is sufficient for use in mass matching protocols, where theoretical mass values of peptides are compared with a linear sequence from a target protein.[36–38] Novel mass values obtained by the subtractive algorithm are used to search out all possible mass matches within the amino acid sequence of the target antigen. Posttranslational modifications can be taken into consideration during these analyses. Those prospective peptide masses matching potential strings within the target antigen (within a tolerance of 0.02% using monoisotopic mass values) are further analyzed. Mass matching is useful because it focuses the ensuing analysis on sequence verification as opposed to complete unknown sequence determination. Because the mass matching protocol described above matches the linear peptide sequence with the experimentally reported mass value, the fragmentation patterns, including all ion types (b, y, a, d, w series), immonium series, and deamidated and dehydrated forms can be mathematically predicted. Thus, peptide masses chosen by mass matching can be sequenced and

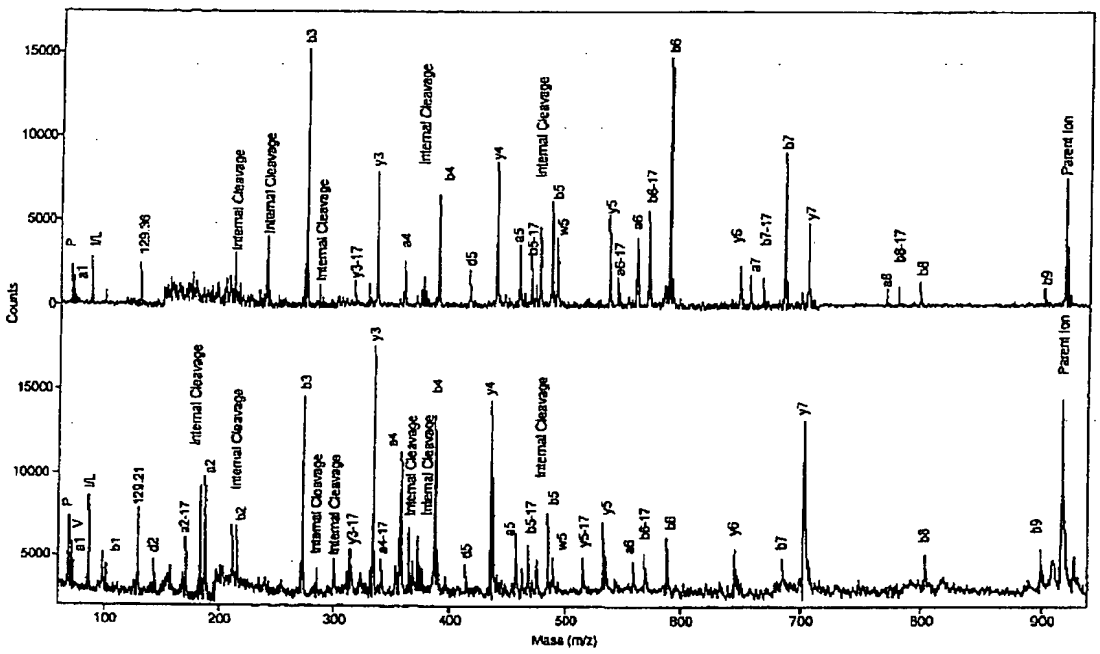

FIGURE 3. Comparison of PSD and CID fragmentation patterns using MALDI-TOF/MS. The upper panel represents the compiled PSD spectra (complete series of mirror ratio stitches) for an HLA-A2 specific peptide derived from an HPV viral protein. Notice the poor fragmentation series below mass 200. The lower panel represents the CID spectra (complete series of mirror ratio stitches) for the same peptide. The lower mass fragments are more pronounced using the CID method. Combining both sets of data promotes a more complete sequence analysis, thus making the determination of an unknown peptide more likely.

the experimentally determined PSD and CID spectra (collected by either MALDI-TOF/MS or ion-trap MS) are compared with the theoretical predicted spectra to verify the mass matching by sequence analysis. Once a candidate peptide is properly identified, synthetic peptide analogues are produced and HPLC retention, mass analyses, and most importantly PSD and CID fragmentation patterns are collected and compared with those used to originally determine the unknown sequence to confirm the unknown sample determination. After verification, the candidate sequence is tested in immunological systems.

III. PEPTIDES AS DRUGS

The ability to determine the precise fragments bound by HLA molecules is a remarkable advance in structural immunology. However, unless these advances can be translated into improved pharmacologic agents they will represent a mere technical feat without broad value. The traditional method for generating cellular immune responses against defined antigens has been to administer full-length proteins or synthetic peptides mixed with adjuvants. As the name implies, adjuvants are used to boost the immunogenicity of these antigens. In many instances these techniques were effective in generating humoral responses, but cellular responses have proven to be more difficult to activate. It has been generally postulated that by administering the precise peptide sequences recognized by T cells, the presentation efficiency and thus potency of these antigens would be increased. In most instances this has not turned out to be the case.[39] The primary obstacles are related to inefficient cell surface binding of the administered peptides and the need for prolonged stability *in vivo*.[40] Synthetic peptides are rapidly degraded by serum peptidases as well as cleared by normal hepatic and renal function.[41] One alternative to using conventional synthetic peptide mixed in adjuvants has been to use modified lipopeptides.[42] These peptides have improved serum stability and use the lipid component to increase cellular permeability. Lipopeptide formulations showed higher potency in animal systems when compared with the standard peptide approach, and human clinical studies are ongoing.[43]

IV. DNA-ENCODED ANTIGENS

Injection of plasmid DNAs encoding antigens into muscle or skin is becoming a well-studied approach to generating immune responses. In this system strong viral promoters are used to overexpress antigens. In some cases, deleterious effects may result from an overabundance of viral antigens and may lead to cell transformation.[44–47] Potential complications arising from this scenario should be avoided if DNA vaccines are to be safely used in humans. One alternative is to use only the naturally processed viral or tumor epitopes to activate T cells. Moreover, providing cells with the pre-processed antigenic peptide bypasses the processing machinery and is a more efficient method in loading HLA molecules with antigen.[48] In addition, by using small fragments as immunogens the potential complications or toxicities associated with the expression of functional proteins are minimized.

Protective immune responses were demonstrated using recombinant vaccinia constructs encoding CTL epitopes.[49–53] However, it has remained unclear whether the coincident immune response to vaccinia virus and the bystander cytokines produced have contributed to the immunogenicity of the antigenic peptides studied. Interestingly, certain palindromic sequences present in some bacterial plasmid DNA stimulate secretion of IL-2 and gamma interferon.[54–58] Cytokines induced in this way amplify T cell reactivity to protein antigens encoded in plasmids[55] and might also provide "help" for a response to preprocessed antigens. Plasmid DNA represents an immunization vehicle that is amenable to repeat injections, does not exempt patients with preexisting immunity to viral vectors, and does not pose risks associated with recombinant viruses.[59] Following intramuscular injection of plasmid DNAs encoding ER-targeted, naturally processed viral epitopes activate CTL responses. We studied this approach in several viral systems, and an example of two are shown in Figure 4. In each model studied, CTL activation was demonstrated; however, to achieve robust activation, this approach requires substantial amounts of plasmid DNA and repeated immunizations. These requirements were surprising because after the first injection muscles expressed

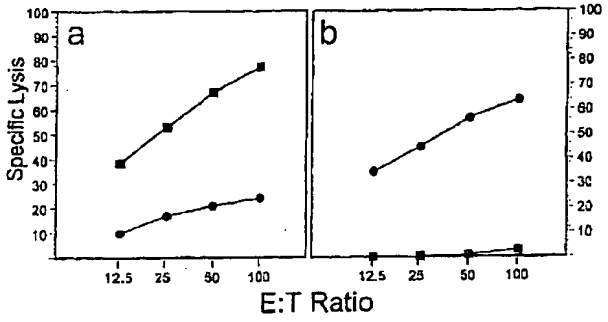

FIGURE 4. Induction of CTL activity following immunization with plasmid DNAs expressing minimal CTL epitopes. Mice were immunized with plasmid DNA encoding leader peptide tagged to amino acids 325 to 332 from the N protein of Sendai virus (panel a) or amino acids 52 to 59 from the N protein of vesticular stomatitis virus (panel b). Animals receiving the SV construct were immunized three times whereas the animals immunized with the VSV construct were only immunized twice. All booster immunizations were performed on 21 d schedules. CTL activity was measured on syngenic targets labeled with either the SV peptide (■) or the VSV (●).

the encoded antigens for extended periods of time (data not shown).

V. FORMULATIONS TO GET DNA INTO CELLS

The primary difficulty in using peptide-based antigens is achieving sufficient binding of HLA molecules. The primary obstacle for nucleic acid-based antigens is getting the DNA inside the cells and, perhaps even more importantly, getting it inside the correct cells. As demonstrated above and in numerous other systems, intramuscular immunization with naked DNA results in activation of immune responses.[59] Until recently, the mechanism by which this occurs was unclear. The theoretical problem has focused on how transfected muscle cells could activate naive T cells given the fact that they do not express co-stimulatory molecules required for initiation of a T cell response. Recent work suggests that the immune response is not initiated by transfected muscle cells but rather by bone marrow derived cells presenting antigen secreted or released from the muscle cells.[60,61]

Another approach was developed in which DNA is coated onto small gold beads which are then introduced through skin by a high pressure ballistic device.[65] This so-called "gene gun" is thought to function by introducing DNA into Langerhans cells that are known to professional antigen presenting cells. This technique requires substantially less DNA than the "naked" DNA approach but does require access to the moderately complicated ballistic device, and it does not disperse DNA systemically which may be desirable in certain therapeutic situations.

More recently an approach was developed to target DNA into professional antigen presenting cells. In this system plasmid DNA is encapsulated into particles composed of polylactide-co-glycolide (PLGA). PLGA microspheres are safe and reliable drug delivery vehicles.[62] Microparticles under ten microns are engulfed by phagocytic cells of the reticuloendothelial system which concentrates encapsulated material within professional APCs.[63] The propensity of polymeric spheres toward APC uptake makes them suited for intracellular delivery of DNA-encoded antigens. These antigens are synthesized by the cell and are accessible to the antigen processing machinery that loads HLA molecules and should accordingly activate T cells.

This new technique results in spheres with a median diameter of 3 to 5 μm. All steps of the procedure have been optimized to reduce DNA shearing and nicking and maintain the supercoiled nature of plasmid DNA. Over 80% is internal as determined by DNAase resistance. To demonstrate that these particles can be ingested and the DNA is released and expressed, a plasmid containing a luciferse cDNA was encapuslated and added to a culture of P388D1 (a murine macrophage cell line). Plasmid DNA expression as measured by luciferase activity is detectable at 24 h post particle ingestion (Table 1). Expression levels continue to increase for 3 d. After 5 d expression begins to decrease, due to cell death from overcrowding under these *in vitro* conditions.

The ability of professional APCs to ingest and express plasmid DNAs contained within microspheres implies that these kinds of vehicles could be used to elicit immune responses *in vivo*.

TABLE 1
Microparticles containing:

	Luciferase DNA	Control DNA
Day 1	1257	103
Day 2	2632	107
Day 3	3400	80
Day 5	763	90

Note: Expression of luciferase in P388D1 cells.

To explore this possibility CTL responses obtained by immunization with encapsulated and unencapsulated plasmid DNAs was compared. An example of these efforts are shown in Table 2. In this particular experiment, mice immunized either one time intraperitoneally with microspheres containing 2 to 5 μg plasmid DNA or immunized twice intramuscularly with a total of 200 μg unencapsulated, naked DNA. These data suggest that administration of encapsulated DNA is more potent than naked DNA, at least when CTL epitopes are used. One of the limitations of using naked DNA clinically is the amount of DNA needed. In the experiments described here, a single injection of microparticles containing ~2 to 5 μg DNA generates stronger responses than two immunizations with unencapsulated DNA using 100 μg in each injection. The increase in efficiency may be due to the protective nature of the polymer coating, but is more likely to result from the increased uptake of DNA by professional APC that are required for activation of naive T cells. Other studies demonstrated that significant immune responses can be generated following injection of DNA loaded microparticles into various routes. Furthermore, synthesis of targeted peptides within cells appears to overcome the limitations of synthetic peptides with respect to T cell activation. In fact, a recent report demonstrates that cells harboring a peptide-expressing DNA construct can produce >50,000 copies of HLA-peptide complexes on the cell surface.[64]

So it would appear that genetic immunization with a minimal amount of information from a viral antigen (i.e., the sequence of a naturally processed peptide) can be used to elicit significant CTL. Furthermore, encapsulation of plasmid DNA into PLGA microparticles increases the potency of DNA delivery over that seen with naked DNA or synthetic peptide and represents a simple and targeted mechanism for reaching antigen presenting cells of the immune system. As it is probable that immunological memory is dependent on residual antigen in lymphoid centers, this type of antigen delivery should ensure a prolonged memory response.[8] The long-term immunological memory to these nucleic acid-based antigens delivered in biocompatible polymers is ongoing.

VI. CONCLUSIONS

The presentation and recognition of antigen has recently been studied at the atomic level. These advances helped to clarify many of the problems encountered in our attempts to regulate the immune recognition. Although much is yet to be learned about the fidelity of T cell receptors, we are well on our way in developing systems to identify and deliver smarter antigens.

TABLE 2

Preparation		% lysis of target cells[a]
Naked BIOTOPE$_{vsv}$	200 μg	14.2 +/– 3.6[b]
BIOTOPE$_{vsv}$ ENSPHERE	2–5 μg	26.7 +/– 3.5[b]

[a] Data is reported as the mean lysis values from three individual measurements at an E:T of 50:1.
[b] Error is reported as the standard deviation; p value < 0.05 as determined by the Students t-test.
Note: Lysis values measured without *in vitro* priming to avoid introducing bias.

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Membrane-Anchored β_2-Microglobulin Stabilizes a Highly Receptive State of MHC Class I Molecules[1]

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The magnitude of response elicited by CTL-inducing vaccines correlates with the density of MHC class I (MHC-I)-peptide complexes formed on the APC membrane. The MHC-I L chain, β_2-microglobulin (β_2m), governs complex stability. We reasoned that genetically converting β_2m into an integral membrane protein should exert a marked stabilizing effect on the resulting MHC-I molecules and enhance vaccine efficacy. In the present study, we show that expression of membranal human β_2m (hβ_2m) in mouse RMA-S cells elevates MHC-I thermal stability. RMA-S transfectants bind an exogenous peptide at concentrations 10^4- to 10^6-fold lower than parental RMA-S, as detected by complex-specific Abs and by T cell activation. Moreover, saturation of the transfectants' MHC-I by exogenous peptide occurs within 1 min, as compared with ~1 h required for parental cells. At saturation, however, level of peptide bound by modified cells is only 3- to 5-fold higher. Expression of native hβ_2m only results in marginal effect on the binding profile. Soluble β_2m has no effect on the accelerated kinetics, but the kinetics of transfectants parallel that of parental cells in the presence of Abs to hβ_2m. Ab inhibition and coimmunoprecipitation analyses suggest that both prolonged persistence of peptide-receptive H chain/β_2m heterodimers and fast heterodimer formation via lateral diffusion may contribute to stabilization. In vivo, peptide-loaded transfectants are considerably superior to parental cells in suppressing tumor growth. Our findings support the role of an allosteric mechanism in determining ternary MHC-I complex stability and propose membranal β_2m as a novel scaffold for CTL induction. ***The Journal of Immunology,*** **2005, 174: 2116–2123.**

To enable meticulous immune surveillance by armed effector CTL, MHC class I (MHC-I)[3] molecules must efficiently bind and present peptides derived from intracellular proteins while minimizing acquisition of undesired peptides from the extracellular milieu. The MHC-I polymorphic H chain (α) is a transmembrane glycoprotein harboring three extracellular domains. The membrane distal α_1 and α_2 domains form the peptide-binding groove, typically capable of accommodating peptides of 8–10 amino acids. The H chain is noncovalently associated, mostly through α3, with the nonpolymorphic β_2-microglobulin (β_2m) L chain, which is a single Ig-like domain, not anchored to the plasma membrane.

The β_2m L chain plays an essential role both in promoting endogenous peptide binding at the endoplasmic reticulum (ER) and in diminishing peptide exchange at the cell surface. To allow binding of peptides transported to the ER from the cytosol by TAP, β_2m must first associate with the H chain to induce a peptide-receptive conformation (1, 2). This heterodimer is unstable and is assembled and subsequently maintained at the peptide loading complex (PLC) in an open state with the concerted guidance of an array of ER chaperones, including calnexin, ERP57, calreticulin, and tapasin. Upon loading with an adequate peptide, the MHC-I molecule assumes a highly stable, closed conformation and is consequently released from the PLC to exit the ER toward the cell surface (for review, see Refs. 3 and 4).

At the cell surface, the ternary MHC-I complex is apparently devoid of auxiliary proteins. Its stability appears to be controlled by an allosteric mechanism, as outlined in detail in a sequence of reports (5–7), and supported by numerous studies, which examined soluble as well as cell-surface MHC-I molecules. According to this model, dissociation of the peptide considerably decreases the affinity of the H chain for β_2m, reducing the heterodimer lifespan to only several minutes or less. Free H chains, in turn, bind the peptide several orders of magnitude weaker than the β_2m-coupled isoform, rendering functional rebinding negligible. Reciprocally, predissociation of β_2m, rather than a peptide from the ternary complex, results in the same affinity decline and fast peptide detachment (8, 9). At the cell surface, the H chain monomer often denatures, shows strong propensity to oligomerize (10), and is later internalized (11). Hence, at physiological conditions in which extracellular concentrations of both peptide and β_2m are limiting, binding an exogenous peptide is an unlikely, although not an improbable, event. In accord with this model are studies (e.g., Refs. 12–17) that showed that loading cells with an extracellular peptide is greatly facilitated in the presence of high concentration of exogenous β_2m.

To evoke CTL, vaccines must target immunogenic peptides to MHC-I molecules on dendritic cells. This can be accomplished extracellularly, either by direct loading or through cross presentation of peptides derived from internalized immunogens, as well as via an endogenous route, which usually entails the use of a genetic approach. The appreciation that β_2m is critical both for ternary complex assembly and for controlling its stability has prompted its

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[3] Abbreviations used in this paper: MHC-I, MHC class I; β_2m, β_2-microglobulin; ER, endoplasmic reticulum; PLC, peptide loading complex; CD3ζ, CD3 ζ-chain; hβ_2m, human β_2m; nβ2m, native hβ_2m; MFI, mean fluorescence intensity.

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incorporation into various vaccine designs. These include recombinant single-chain β_2m/H chain MHC-I dimers (18–22), soluble (23–25) or cellular (23, 26) peptide/β_2m fusions, and cell-expressed single-chain peptide/β_2m/H chain trimers (27, 28).

We recently generated membrane-anchored chimeric β_2m/peptide molecules fused with the intracellular activation domain of CD3 ζ-chain (CD3ζ). These polypeptides functionally associate with endogenous MHC-I H chains in transfected T cells and yield an exceedingly high complex density on the cell surface (29). We reasoned that attachment of β_2m to the cell membrane offers a unique, universal tool for the generation and stabilization of immunogenic MHC-I/peptide complexes and may serve as a novel vaccine platform. As a preparatory step in the design and evaluation of CTL-inducing vaccines based on membrane-anchored β_2m, we investigated biochemical and functional outcome of its expression in RMA-S cells.

Materials and Methods

Vectors and expression plasmids

The use of the pBJ1-Neo expression vector and the construction of plasmid 21-2, encoding chimeric human β_2m (hβ_2m)/CD3ζ, have been described elsewhere (29).

In plasmid 323-5, the CD3ζ intracellular domain was replaced with that of H-2K^b to encode hβ_2m/K^b as follows: first-strand DNA synthesis from mRNA prepared from RMA cells was performed with the reverse primer 5′-CGCGCGGCCGCAAGTCCACTCCAGGCAGC-3′, and PCR was then conducted by adding the sense primer 5′-CCCTCGAGCTCCACT GTCTCCAACATGGCG-3′. The product, encoding the 3′-part of the peptide bridge and H-2K^b transmembrane and cytoplasmic portion, was cloned into pBJ1-Neo as an *XhoI/NotI* fragment, together with the *XbaI/XhoI* fragment from plasmid 21-2, encoding hβ_2m leader, mature protein, and 5′-part of the peptide bridge.

Expression plasmid 845-6(nβ_2m) encodes full-length, native hβ_2m (nβ_2m), which was amplified from Jurkat cells mRNA by reverse transcription-PCR with the sense primer 5′-GGGTCTAGAGCCGAGAT GTCTCGCTCCGTG-3′ and the reverse primer 5′-CGCGCGGCCGCT TACATGTCTCGATCCCACTTAAC-3′ and inserted into pBJ1-Neo as a *XbaI/NotI* fragment.

Mice and cell lines

Eight to 12-wk-old C57BL/6 mice were purchased from The Jackson Laboratory and bred at the Weizmann Institute of Science facilities. Animals were maintained and treated according to the Weizmann Institute of Science animal facility and National Institutes of Health guidelines.

RMA is a Rauscher virus-transformed lymphoma cell line of C57BL/6 (H-2^b) origin, and RMA-S is a RMA TAP-deficient mutant (30). Cells were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 50 μm 2-ME. B3Z (31), an OVA$_{257-264}$-specific, K^b-restricted CTL hybridoma, which expresses the NFAT-LacZ reporter gene, was a kind gift from Dr. N. Shastri (University of California, Berkeley, CA). MO5 is an OVA gene-transfected B16 murine melanoma clone of C57BL/6 origin. These cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids (Sigma-Aldrich), combined antibiotics, and 500 μg/ml G418 (both from Invitrogen Life Technologies).

Abs, proteins, and peptides

25-D1.16, a mAb specific to K^b-OVA$_{257-264}$ (32), was a kind gift from Dr. R. Germain (National Institutes of Health, Bethesda, MD). mAb against hβ_2m (clone BM-63) was from Sigma-Aldrich. Polyclonal rabbit anti-hβ_2m Ab was from DakoCytomation. mAbs 20-8-4 and Y3, specific to H-2K^b, and 28-14-8, specific to H-2D^b, were purified from hybridoma supernatants. Purified hβ_2m was from Sigma-Aldrich. OVA$_{257-264}$ was synthesized by Dr. M. Fridkin (Weizmann Institute of Science, Rehovot, Israel).

DNA transfection

A total of 0.8 ml of 4 × 10^6 RMA-S cells/ml was mixed in a 4-mm sterile electroporation cuvette (ECU-104; EquiBio) with 20 μg of *SalI*-linearized plasmid DNA. Transfection was performed with an Easyject Plus electroporation unit (EquiBio) at 350 V, 750 μF. Cells were resuspended in fresh medium and cultured for 24–48 h in 96-well plates before addition of G418 to 1 mg/ml. Resistant clones were expanded in 24-well plates and screened by flow cytometry for expression of hβ_2m.

Flow cytometry

A total of 10^6 cells was washed with FACS buffer (PBS, 5% FCS, and 0.05% sodium azide) and incubated for 30 min on ice with 100 μl of first (or control) Ab at 10 μg/ml. Cells were then washed and incubated on ice with 100 μl of 1/100 dilution of goat anti-mouse IgG (Fab-specific)-FITC-conjugated polyclonal Abs (Sigma-Aldrich) for 30 min, washed, resuspended in PBS, and analyzed with FACSCalibur (BD Biosciences). Mean fluorescence intensity (MFI) was calculated using CellQuest software (BD Biosciences). Quantitative analysis of cell surface Ags was performed with QIFIKIT (DakoCytomation) according to the manufacturer's instructions.

Peptide loading

Twenty-four hours before onset of the experiment, cells were washed three times with PBS and incubated in OptiMEM serum-free medium (Invitrogen Life Technologies) at 37°C. Cells were then transferred to fresh OptiMEM medium and coincubated with a peptide in 24-well plates at 1 × 10^6 cells/ml.

B3Z activation

Peptide-pulsed or control cells were washed three times with PBS, resuspended in nonselective medium at 5 × 10^5 cells/ml, and 50 μl of the cells were added to microtiter plates in triplicates. PBS-washed B3Z cells were resuspended in fresh growth medium at 5 × 10^5 cells/ml, added to wells at a 1:1 ratio, and coincubated for 6 h at 37°C. Cells were washed twice with PBS and fixed with 0.25% glutaraldehyde 5 min at 4°C, washed three times with PBS, incubated overnight with 100 μl of 5-bromo-4-chloro-3-indolyl β-D-galactoside solution (0.2% X-Gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, and 5 mM $K_3Fe(CN)_6$ in PBS), and scored under the microscope for blue staining.

Immunoprecipitation

A total of 7 × 10^7 cells were harvested and washed twice with PBS. The cells were incubated with 15 μg of Ab for 2 h at 37°C. Cells were then washed three times with PBS, resuspended in cold lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), and incubated for 25 min on ice with gentle agitation. Cell lysate was collected by centrifugation (14,000 rpm, 5 min, 4°C). Thirty microliters of protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) were washed twice in cold lysis buffer and incubated with the cell lysate for 2 h at 4°C with gentle agitation. Nonbound proteins were removed by five washing cycles with lysis buffer (10,000 rpm, 1 min, 4°C). The precipitate was then eluted from the beads using elution buffer (0.1 M glycine (pH 2.7)) and immediately neutralized with 2 M Tris (pH 9). Recovered proteins were kept at −20°C.

Immunoblot analysis

Protein samples were boiled for 3 min, separated on a 10% nonreducing SDS polyacrylamide gel at 50 mA, and transferred onto a nitrocellulose membrane. The membrane was blocked with milk buffer (0.3 g of Na_2HPO_4, 2.19 g of NaCl, 25 ml of double-distilled water, and 225 ml of 1% low-fat milk) overnight at 4°C and 1 h at room temperature, washed twice with PBS, and incubated for 2 h with the primary Ab. The membrane was then washed 6 times for 6 min each with PBS, incubated for 1 h with the secondary peroxidase-conjugated Ab, washed 6 times for 6 min each with TPBS (PBS with 0.05% Tween 20), 6 times for 6 min each with PBS, and then developed using chemiluminescence kit (Pierce) and x-ray film 100NIF (Fuji).

Tumor immunotherapy

Ten mice in each experimental group were inoculated s.c. in the upper back with 10^5 MO5 cells/mouse. Local tumor diameter was measured with calipers. Starting 8 days later, when the tumor reached 3–4 mm in diameter, mice were immunized i.p. four times at 7-day intervals with 2 × 10^6 irradiated peptide-loaded cells, which were prepared as follows: RMA-S cells and transfectants were washed, resuspended at 5 × 10^6/ml in OptiMEM, and incubated with 50 μM synthetic peptide for 2 h at 26°C and then for 4 h at 37°C. The samples were irradiated (5000 rad) and washed. Mice were monitored daily and sacrificed when moribund. Survival was defined as the day when mice were sacrificed.

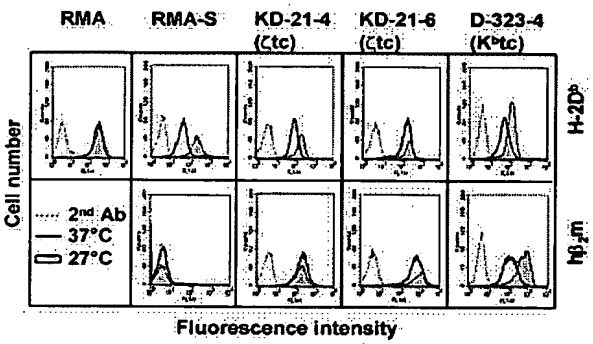

FIGURE 1. FACS analysis of transfectants. RMA, RMA-S, KD-21-4(ζtc), KD-21-6(ζtc), and D-323-4(K^{b}tc) cells were grown in serum-free medium for 24 h at 27 and 37°C. Cells were then incubated with anti-H-2D^b (28-14-8) and anti-hβ_2m (BM-63) mAbs, or no Ab as negative control, and then with FITC-conjugated goat anti-mouse Fab Abs and analyzed by FACS. Fluorescence intensity is presented in logarithmic scale.

Results

Construction of hβ_2m harboring two different membranal anchors

The original design of membrane-anchored hβ_2m has already been delineated (29). Briefly, the carboxyl-terminal methionine residue of hβ_2m was genetically linked to a peptide bridge, comprising the 13 membrane-proximal amino acids of the extracellular portion of HLA-A2, LRWEPSSNPTIPI (single-letter code), which encompasses the proline-rich connecting peptide. This sequence was tethered to the amino terminus of the transmembrane segment of the mouse CD3ζ to include the entire intracellular domain (hβ_2m/CD3ζtc). At the TCR complex, CD3ζ primarily forms disulfide-bridged homodimers through the transmembranal cysteine residue. Indeed, although associated with endogenous MHC-I H chains in transfected T cells, these chimeric hβ_2m/CD3ζtc polypeptides homodimerize (D. Berko, G. Cafri, and A. Margalit, unpublished observations). To rule out possible contribution of dimerization artifacts to the activity of hβ_2m/CD3ζtc, we have assembled a similar construct, substituting the CD3ζ transmembrane and cytoplasmic portion with that of H-2K^b (hβ_2m/K^{b}tc).

Expression of membranal β_2m in RMA-S increases MHC-I thermal stability

RMA-S cells do not express functional TAP, and their MHC-I assembly pathway is thus cut from its major peptide supply. As a result, MHC-I molecules are mostly loaded with scarce, suboptimal peptides generated at the ER, and their surface level at 37°C is substantially reduced compared with RMA cells. However, this level can be elevated by incubating cells with high-affinity peptides or at lower temperatures (26–28°C), in which these peptide-receptive molecules are stabilized. We were interested in testing whether the mere association with membranal β_2m could stabilize these thermally labile MHC-I molecules. Three RMA-S transfectants were generated: KD-21-4(ζtc) and KD-21-6(ζtc), expressing a relatively moderate and high level of hβ_2m/CD3ζ, respectively, and D-323-4(K^{b}tc), expressing the hβ_2m/K^{b}tc construct. Fig. 1 shows that all three clones, but not the parental cells, stain brightly for hβ_2m. Surface level of H-2D^b at 37°C, which drops in RMA-S ~1 log compared with 27°C or RMA, is almost completely restored in KD-21-6(ζtc), and to somewhat lesser, still noticeable, extent in KD-21-4(ζtc) and D-323-4(K^{b}tc). Interestingly, clones KD-21-6(ζtc) and D-323-4(K^{b}tc) express more surface hβ_2m at 27°C than at 37°C unlike clone KD-21-4(ζtc), which displays comparable levels at both temperatures.

We went on to obtain a quantitative evaluation of the dual effect of hβ_2m expression and exogenous peptide binding on the level of MHC-I molecules in RMA-S cells. For this purpose, we used a commercial kit for quantitative analysis of cell-surface Ags with mouse Abs, and we measured binding of the synthetic chicken OVA peptide OVA$_{257-264}$ to H-2K^b, using the K^b-OVA$_{257-264}$ complex-specific 25-D1.16 mAb. This analysis is summarized in Table I. Expression of hβ_2m elevates the level of H-2K^b 5- to 8.5-fold, whereas addition of the peptide results in an additional, relatively moderate increase of 1.3- to 1.5-fold of H-2K^b, with no effect on H-2D^b. In comparison, incubation of RMA-S with a peptide under these conditions raised the surface level of H-2K^b 4-fold. The somewhat weaker effect of hβ_2m expression on the level of H-2D^b (1.9- to 5.7-fold increase) is in agreement with the lower ability of hβ_2m to stabilize cell surface H-2D^b compared with K^b (33).

Membranal β_2m greatly augments the ability of RMA-S to bind an exogenous peptide

We then examined whether these thermally stable MHC-I molecules possess enhanced ability to bind synthetic OVA$_{257-264}$. Fig. 2*A* depicts a typical binding experiment. Threshold for detection of OVA$_{257-264}$ binding to RMA-S was at a concentration of 1 ng/ml, which is in good agreement with a previous report (32). However, significant peptide binding to KD-21-6(ζtc) was evident even at 1 pg/ml, and residual binding at this concentration could also be detected in D-323-4(K^{b}tc). Such results could in fact be attributed both to the overall increase in the level of β_2m expressed by these cells and to the higher affinity of hβ_2m than mouse β_2m for H-2K^b

Table I. *Absolute numbers of cell surface MHC-I molecules expressed on RMA-S cells and transfectants, with or without OVA$_{257-264}$ (Pep)*

Cell[a]	Anchor	Peptide[b]	hβ_2m[c] (BM-63)	H-2D^b (28-14-8)	H-2K^b (20-8-4)	K^b-pep (25-D1.16)
RMA-S		−		33,925	15,313	
RMA-S		+		23,964	60,034	39,201
KD-21-6	CD3ζ	−	320,762	64,699	127,375	
KD-21-6		+	353,471	70,213	203,623	110,395
KD-21-4	CD3ζ	−	129,766	65,846	78,297	
KD-21-4		+	141,223	61,838	114,630	
D-323-4	H-2K^b	−	564,946	194,956	131,260	
D-323-4		+	616,605	186,015	177,336	101,605

[a] RMA-S transfectants expressing hβ_2m with the indicated anchors were grown 24 h in serum-free medium before peptide loading.

[b] Peptide was loaded at 2 μg/ml for 2 h.

[c] All mAbs used in this assay are mouse IgG. Analysis was performed by FACS with QIFIKIT (DakoCytomation), using a set of calibrating beads precoated with an average of 0, 3,600, 16,000, 53,000, 218,000, and 620,000 mouse IgG molecules/bead.

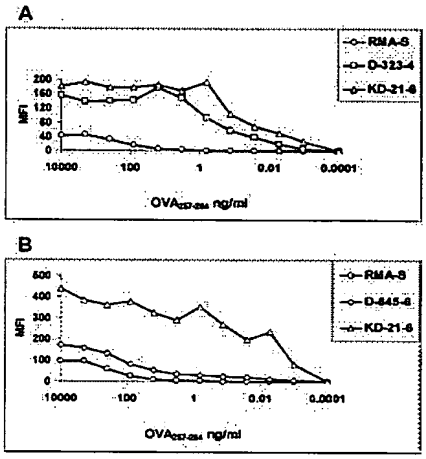

FIGURE 2. Analysis of peptide binding by transfectants. *A*, Comparison of KD-21-6(ζtc) and D-323-4(K^btc) with parental RMA-S cells. *B*, Comparative analysis of KD-21-6(ζtc) against 845-6(nβ_2m) and RMA-S. For both experiments, indicated cells were grown at 37°C for 24 h in serum-free medium and were then incubated for 2 h with serial dilutions of synthetic $OVA_{257-264}$. Cells were stained with the anti-H-$2K^b$-$OVA_{257-264}$ mAb 25-D1.16, and FACS analysis was performed with FITC-conjugated anti-mouse Fab Abs. MFI was calculated using the CellQuest FACS program.

(33, 34). To test this possibility, we transfected RMA-S cells with nβ_2m. Unlike membranal β_2m, which is expected to be present on the cell surface also as a noncoupled monomer and reach very high density, level of nβ_2m entirely depends on available MHC-I H chains. To carry out a reliable comparison of peptide loading, we screened a large number of transfectants for the highest expresser and chose clone D-845-6(nβ_2m). Preliminary analysis of thermal stability of H-$2K^b$ and D^b on the surface of these cells showed a phenotype resembling that of the parental RMA-S cells, rather than KD-21-6(ζtc) or D-323-4(K^btc) (data not shown). Fig. 2*B* indeed reveals a slight increase in the ability of D-845-6(nβ_2m) to bind $OVA_{257-264}$ and in its saturation level compared with RMA-S cells, but it is still significantly lower than for KD-21-6(ζtc).

Such a dramatic increase in the ability to bind an exogenous peptide is of particular relevance to the design of cell vaccines expressing membrane-anchored β_2m. Therefore, we evaluated the sensitivity with which a peptide-specific T cell hybridoma can respond to transfectants vs parental cells following peptide loading. Fig. 3 presents the peptide dose response of the K^b-restricted, $OVA_{257-264}$-specific B3Z hybridoma (31). In good agreement with the FACS analysis, B3Z cells could be activated by KD-21-6(ζtc) and D-323-4(K^btc) cells pulsed with as little as 1 and 100 fg/ml of the peptide, respectively, whereas RMA-S cells charged with as high as 1 ng/ml failed to activate these cells detectably under the same experimental conditions.

Membranal β_2m increases on-rate of peptide binding

We next asked whether this marked effect on peptide binding is also manifested in a significant change in binding kinetics. To address this question, we designed a flow cytometry-based binding assay, which scores binding of the peptide at a saturating concentration following incubation for different time intervals. Results are shown in Fig. 4*A* and indicate that saturation of transfectant KD-21-6(ζtc) and near saturation of D-323–4(K^btc) are already achieved within 1 min, whereas D-845-6(nβ_2m) and RMA-S require 1 and 2 h, respectively. To assess peptide dissociation rates,

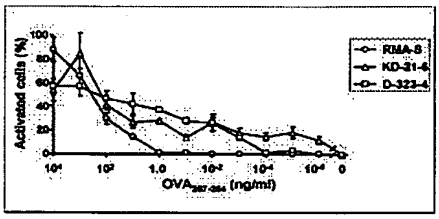

FIGURE 3. Dose-dependent response of the B3Z T cell hybridoma to transfectants KD-21-6(ζtc) and D-323-4(K^btc) and parental RMA-S cells loaded with $OVA_{257-264}$. Indicated APC were grown for 24 h in serum-free medium and incubated for 2 h with serial dilutions of the peptide. Peptide-loaded cells were then incubated overnight in triplicates at a 1:1 ratio (4×10^5 cells each) with B3Z cells, a CTL hybridoma specific to the $OVA_{257-264}$-H-$2K^b$ complex, which expresses the NFAT-LacZ reporter gene. T cell activation was monitored by intracellular 5-bromo-4-chloro-3-indolyl β-D-galactoside staining. Percentage of cells stained blue was evaluated under a light microscope and scored as an average of 16 fields. Fifty-four percent of B3Z cells were activated with the anti-TCR Ab 2C11 (anti-CD3ϵ, data not shown). As activation by transfectants was conducted at 1:1 ratio, a score of 27% activated B3Z cells was considered 100% of potentially activated cells, and all results were normalized accordingly.

we incubated cells with a saturating amount of peptide and similarly followed persistence of specific complexes on the cell surface. Fig. 4*B* shows no significant difference in dissociation rate between KD-21-6(ζtc), D-323-4(K^btc), and RMA-S.

Exogenous β_2m enhances peptide binding by RMA-S but not transfectants

We considered the possibility that the chimeric β_2m polypeptide completely saturates MHC-I H chains on the surface of transfected cells. In this case, addition of exogenous β_2m would only have a negligible effect on the kinetics of peptide binding to these clones

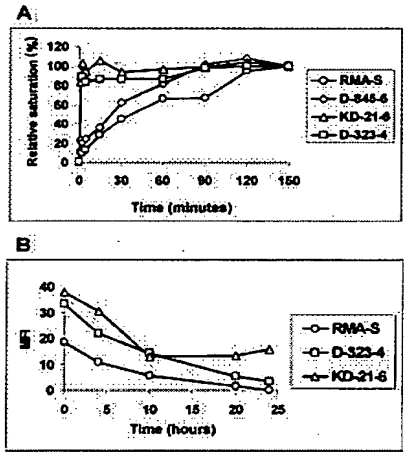

FIGURE 4. Peptide-binding kinetics of transfectants KD-21-6(ζtc), D-323-4(K^btc), and 845-6(nβ_2m). *A*, Cells were grown at 37°C for 24 h in serum-free medium and were then incubated at 37°C for different time intervals with 2 μg/ml $OVA_{257-264}$. After incubation, cells were washed several times and stained with the complex-specific mAb 25-D1.16. FACS analysis was performed with anti-mouse FAB-FITC, and MFI was derived. One hundred percent saturation was determined as the MFI value obtained after 150 min of incubation with the peptide. *B*, Cells were incubated for 2 h with 2 μg/ml $OVA_{257-264}$ in the presence of serum, washed, and incubated at 37°C in complete medium for 24 h. After each time, interval cells were washed and fixed with 0.5% paraformaldehyde. All samples were stained and analyzed in parallel as in *A*.

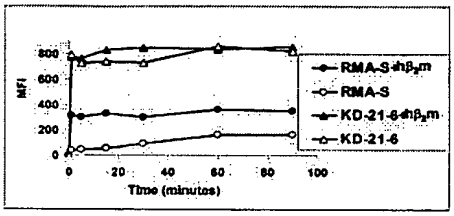

FIGURE 5. Effect of exogenous hβ_2m on peptide-binding kinetics. KD-21-6(ζtc) and parental RMA-S cells were grown at 37°C for 24 h in serum-free medium and were then incubated in the presence and in the absence of 10 μg/ml purified nβ_2m for 1 h at 37°C. Cells were then incubated for different time intervals with 2 μg/ml $OVA_{257-264}$ at 37°C. After incubation, cells were washed three times and were then stained with the complex-specific 25-D1.16 mAb and analyzed by FACS.

but is expected to substantially increase on-rate of binding to the parental cells. To test this assumption, we used the experimental design of the peptide-binding assay but now examined the effect of preincubation of cells in the presence of a high concentration of soluble hβ_2m. Indeed, Fig. 5 shows no effect on KD-21-6(ζtc) but demonstrates a striking increase in the on-rate of binding to RMA-S, which now appears similar to that of the transfectant, so that saturation occurs within the first minute of incubation with the peptide.

Abs to hβ_2m inhibit peptide binding to cells expressing membranal β_2m

Results obtained so far imply that availability of β_2m on the cell surface is a key factor, which governs the unique peptide-binding properties of the transfected cells. To assess the contribution of membranal β_2m directly, we used polyclonal Abs to hβ_2m. When applied along with an exogenous peptide for even a short period, these Abs should significantly diminish de novo formation of heterodimers but would have a smaller effect on pre-existing ones. In

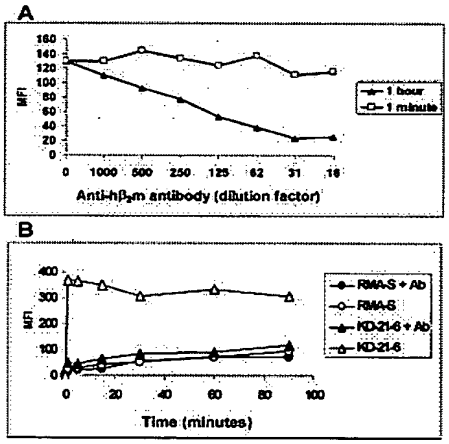

FIGURE 6. Effect of anti-hβ_2m Abs on peptide-binding kinetics. *A*, KD-21-6(ζtc) cells were grown at 37°C for 24 h in serum-free medium and then incubated with serial dilutions of polyclonal rabbit anti-hβ_2m Ab (DakoCytomation), either for 1 h at 37°C or for 1 min at room temperature. Cells were then incubated with 2 μg/ml $OVA_{257-264}$ for 1 min, washed, stained with 25-D1.16, and analyzed by FACS. *B*, KD-21-6(ζtc) and parental RMA-S cells were similarly grown and incubated in the presence or in the absence of 1/62 dilution of the anti-hβ_2m Ab for 1 h at 37°C. Cells were then incubated for different time intervals with 2 μg/ml $OVA_{257-264}$ at 37°C, washed, and similarly analyzed by FACS.

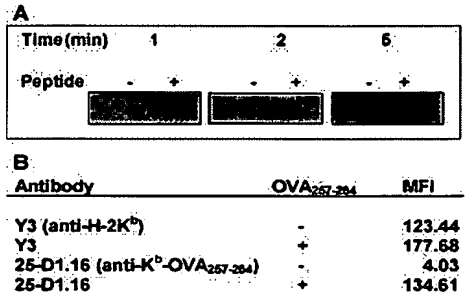

FIGURE 7. Coimmunoprecipitation of hβ_2m with surface H-2K^b molecules from D-323-4(K^{b}tc) cells. Cells were grown 24 h in serum-free medium and incubated for 2 h with (+) or without (−) 2 μg/ml $OVA_{257-264}$. *A*, Y3 Ab was incubated with cells for 2 h, cells were washed and lysed with 1% Nonidet P-40, and immune complexes were immunoprecipitated with protein G-Sepharose beads. Immunoblots of eluted samples were analyzed by ECL using HRP-conjugated rabbit anti-hβ_2m polyclonal Abs. Results of three exposures times (minutes) are shown. Under these experimental conditions, capturing of intracellular H-2K^b molecules is minimized but cannot be entirely ruled out. *B*, FACS analysis of the same cells in the same experiment, performed with the indicated Abs. Results are shown as MFI.

contrast, longer preincubation with the Abs is expected to result in a more pronounced effect also on pre-existing heterodimers. Fig. 6*A* presents flow cytometry analysis of the amount of peptide bound to KD-21-6(ζtc) cells following 1 min of incubation with a saturating amount of peptide in the presence of different dilutions of the Abs: in one set, the Ab was added to the assay along with the peptide, and in the other set, the Abs were preincubated for 1 h. These results suggest that the faster kinetics can largely be attributed to pre-existing heterodimers because only a minimal effect is evident following short incubation. Longer preincubation with Ab, nevertheless, severely diminishes binding, implying reformation of heterodimers is important. We then monitored ternary complex formation, following 1 h of preincubation with an effective concentration of these inhibiting Abs. Fig. 6*B* shows that the increase in binding on-rate is completely abolished under these conditions, and the peptide-binding profile is practically identical with that of RMA-S cells, which, as expected, are unresponsive to the presence of these Abs.

Peptide binding primarily recruits pre-existing membranal β_2m molecules

To obtain direct evidence for the involvement of membranal β_2m in peptide binding, we performed a coimmunoprecipitation analysis of transfectant D-323-4(K^{b}tc), using the anti-H-2K^b Ab Y3. This experiment compared the amount of coprecipitated hβ_2m before and after peptide loading, relative to the increase in total surface H-2K^b during the incubation period, as monitored by FACS. Results presented in Fig. 7*A* reveal a vast increase in the amount of hβ_2m coprecipitated with H-2K^b H chain from the cell membrane following peptide binding, which is accompanied by only a 44% increase of total H-2K^b (Fig. 7*B*).

Transfectants are superior to parental cells in treating tumor-bearing mice

To demonstrate the potential of membrane-anchored β_2m as a new platform for CTL induction in vivo, we performed a tumor suppression experiment. MO5 is a transfectant of the B16 melanoma cell line (H-2^b), which expresses chicken OVA and presents $OVA_{257-264}$ in the context of H-2K^b. C57BL/6 mice were challenged with 10^5 MO5 cells each. Starting 8 days later, mice were

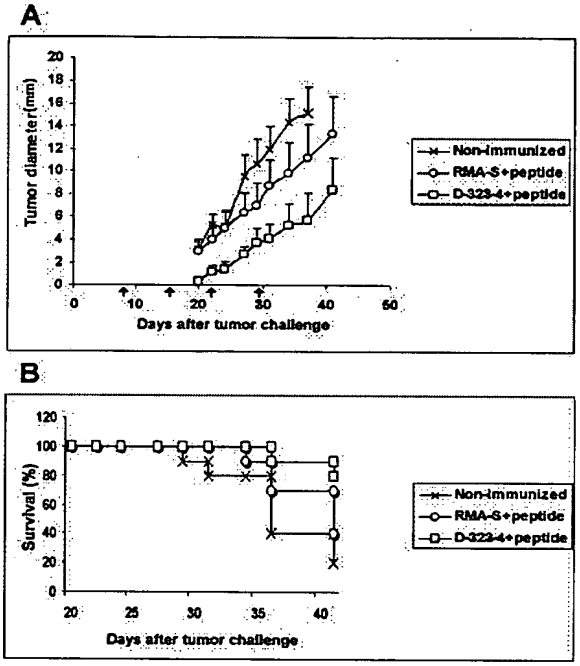

FIGURE 8. Inhibition of tumor growth by peptide-pulsed D-323-4(K^btc) and RMA-S cells. Ten C57BL/6 mice in each experimental group were challenged with MO5 tumor cells. Eight days later, mice were vaccinated four times at 7-day intervals with D-323-4(K^btc) or RMA-S cells pulsed with a saturating concentration of $OVA_{257-264}$ or with no cells, as described in *Materials and Methods*. *A*, Tumor progression during 6 wk after tumor challenge expressed as mean tumor diameter in each of the three groups. Arrows indicate immunizations. *B*, Survival curves of mice in the three groups during the same period. Mice were monitored daily and sacrificed when moribund, which occurred when tumor reached a diameter of ~20 mm.

subjected to an immunization regimen with either irradiated D-323-4(K^btc) or parental RMA-S cells, both pulsed with $OVA_{257-264}$, or with no cells as control. Tumor growth was substantially delayed in mice vaccinated with D-323-4 compared with RMA-S (Fig. 8*A*), and 80% of mice in the D-323-4(K^btc) vaccinated group remained alive (five tumor-free), compared with only 40% (three tumor-free) in the group immunized with RMA-S 6 wk after the tumor challenge (Fig. 8*B*).

Discussion

In this study, we expressed membrane-anchored hβ_2m in mouse RMA-S cells and showed up to 10^6-fold increase in the ability of transfected cells to bind an exogenous peptide and at least 50-fold faster binding kinetics. The affinity of β_2m for the MHC-I H chain determines heterodimer stability and, as a result, peptide-binding capacity (35, 36). Indeed, hβ_2m possesses a higher affinity for most mouse MHC-I H chains than does mouse β_2m, including H-2K^b and H-2D^b, which are expressed by RMA-S (33, 34). Hence, these observations could be solely attributed to the elevated level of β_2m expressed by transfected cells and to the higher affinity of the human L chain for MHC-I H chains. However, several findings suggest a major contribution of yet another factor. First, the combined effect of affinity with expression level, as revealed by the quantitative FACS analysis (Table I), mounts, at most, to only 8.5-fold increase of cell surface H-2K^b in transfectants, a value that is too small to account for the magnitude of the observed phenomena. Second, clone D-845-6(nβ_2m), which expresses nβ_2m, exhibits only a mild shift in peptide-binding profile (Figs. 2 and 4) compared with transfectants expressing the membranal derivative. Third, at peptide saturation, the total level of H-2K^b-$OVA_{257-264}$ complexes formed on the surface of transfectants is only 3- to 5-fold higher than on parental RMA-S cells (Figs. 2 and 4–6).

We repeatedly observed higher level of membrane-anchored hβ_2m at 27°C than at 37°C (Fig. 1). This difference may reflect a remaining degree of thermal instability of MHC-I molecules expressed by transfectants, and suggests that exit of membranal hβ_2m to the cell surface from the ER still depends on available peptides.

A large fraction of MHC-I molecules on RMA-S cells cultured at 37°C are either H chain monomers or short-lived heterodimers (9, 37) so that efficient peptide binding requires equilibrium with exogenous β_2m. Of particular importance is the observation that D-845-6(nβ_2m) cells reach saturation only after 1 h of incubation with the peptide, whereas RMA-S cells are fully saturated within 1 min in the presence of soluble hβ_2m. This result indicates that the contribution of exogenous β_2m to peptide-binding kinetics far exceeds the effect exerted by cellular expression of nβ_2m. Together with the similar binding kinetics monitored for RMA-S in the presence of β_2m and for KD-21-6(ζtc), our data imply that it is the membranal anchorage of β_2m, which is primarily responsible for the pronounced effect on transfected cells. The ability of transfectants to functionally bind an exogenous peptide at concentrations up to 10^6-fold lower than RMA-S cells supports this conclusion. It coincides with the notion that peptide binding to β_2m/H chain heterodimers occurs at far lower concentrations than to free H chains (38, 39), which is probably the prevalent species in RMA-S.

Several nonmutually exclusive explanations may account for this new phenotype. First, the appended polypeptide sequences form additional contacts with the H chain in a manner, which secures a receptive conformation of the peptide-binding groove. One of the conjectures of the allosteric model (6, 7) is that the empty heterodimers exist in two different conformations, only one of which is capable of binding a peptide. Conversion to the active state constitutes a rate-limiting step in the binding reaction. Although our experiments do not directly address this possibility, the accelerated kinetics of RMA-S supplied with native exogenous β_2m seem to preclude a major role for such an effect. Second, membrane anchorage of β_2m stabilizes empty heterodimers and substantially prolongs their persistence on the cell surface in a peptide-receptive conformation. It is interesting to note in this regard that an additional membrane anchor is in fact provided to the β_2m/H chain dimer at the PLC by tapasin, which may also contribute to stability of the open conformation. Third, whereas following dissociation from the MHC-I complex nβ_2m is practically lost from the cell, its membranal attachment retains it in the cell membrane so that it is capable of rebinding to H chains by lateral diffusion. This is a highly likely scenario, which is supported by the observation that preincubation of KD-21-6(ζtc) cells with Abs to hβ_2m for 1 h severely impairs their peptide-binding capacity, reducing it to that of parental RMA-S cells (Fig. 6*B*). Although disruption of only partially and transiently denatured dimers cannot be ruled out, Ab blocking of β_2m rebinding to form peptide-receptive heterodimers is a conceivable interpretation of this result. The half-life of the empty heterodimer formed between H-2K^b and nβ_2m has been estimated to be in the order of several minutes (6, 7). The finding that saturation by peptide is achieved already within 1 min sets an upper limit on the lifespan of noncoupled H

chains. Taken together, these data imply that in the excess of membranal β_2m and in the absence of peptide, at any given time point the majority of H chains are present on the transfectants cell surface as heterodimers rather than noncoupled monomers. Hence, the marked increase in the amount of chimeric hβ_2m coimmunoprecipitated with H-2K^b in the presence of peptide (Fig. 7*A*) may primarily reflect the corresponding increase in affinity for the H chain, which prevents heterodimer disruption by the detergent, rather than recruitment of β_2m by lateral diffusion. In fact, both the second and third accounts predict markedly elevated persistence of preformed hβ_2m/H chain heterodimers at the cell surface, and in this regard, they are indistinguishable. Collectively, our findings provide yet another demonstration of the allosteric control underlying MHC-I stability. Genetic input of β_2m with high accessibility to MHC-I H chains results in marked stabilization of a peptide-receptive conformation, as manifested both by vast acceleration of binding kinetics and the ability to bind a peptide at extremely low concentrations.

Although the level of hβ_2m expressed by KD-21-6(ζtc) cells is comparable to that of D-323-4(K^{b}tc) cells, the former display a more pronounced phenotype. In particular, they functionally present synthetic peptide to B3Z T cells at a concentration at least 100-fold lower than D-323-4(K^{b}tc), as shown in Fig. 3. In fact, under the experimental conditions used in this experiment, a concentration of 1 fg/ml peptide translates into an average of ~2.5 peptides/cell. Another interesting finding is the plateau reached during dissociation after 10 h (Fig. 4*B*), which was observed for KD-21-6(ζtc) but not for D-323-4(K^{b}tc). The observation was reproducible and may reflect binding equilibrium with the peptide released into the culture medium in the course of incubation, achieved as a result of the enhanced ability of KD-21-6(ζtc) cells to bind the peptide at exceedingly low concentrations. We were unable to detect binding of the complex-specific Ab in other experiments performed in the presence of serum and in the absence of synthetic peptide (data not shown). This rules out contribution of cross-reactive peptide(s) from either an exogenous or an endogenous source to this observation. We tend to attribute this difference to the natural propensity of the CD3ζ-derived anchor in KD-21-6(ζtc) to homodimerize, which may confer yet greater stability on the resulting MHC-I molecules.

The properties endowed on MHC-I molecules by expression of membrane-anchored β_2m bear obvious implications to vaccine development. Two versions of this polypeptide can be envisaged. The first is the one described in this study, namely, a peptide-less mode. Introduction of the gene into dendritic cells is expected to endow them with the capacity to be effectively loaded in vivo or ex vivo with a desired combination of immunogenic peptides, applied at increasingly low concentrations. The fact that β_2m is monomorphic and can pair with all human MHC-I alloforms renders the use of such a construct universal in essence. The preliminary in vivo evaluation of this design with the MO5 tumor model described in this report (Fig. 8) underscores the potential advantages of this modality in stimulating CTL for tumor immunotherapy. The second approach is to genetically fuse an antigenic peptide to the amino terminus of membranal β_2m via a synthetic linker (see Refs. 23–29). This strategy combines the overriding of bottlenecks associated with the conventional processing and presentation pathway with the stabilization effect described herein, while requiring only one expression cassette for all immunogens. In vivo studies indeed show that cells expressing such genes are superior to cells saturated with synthetic peptides in their ability to stimulate CTL generation (A. Margalit et al., manuscript in preparation).

In summary, the effect of membrane-anchored β_2m on the resulting MHC-I molecules strongly supports the allosteric model for ternary complex formation. This β_2m derivative offers a highly sensitive tool for studying peptide loading onto MHC-I molecules and provides a novel and widely applicable genetic platform for CTL induction.

Acknowledgments

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Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells

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VIRUS-infected cells can be eliminated by cytotoxic T lymphocytes (CTL), which recognize virus-derived peptides bound to major histocompatibility complex (MHC) class I molecules on the cell surface[1,2]. Until now, this notion has relied on overwhelming but indirect evidence, as the existence of naturally processed viral peptides has not been previously reported. Here we show that such peptides can be extracted from virus-infected cells by acid elution. Both the naturally processed H-2-D^b-restricted and H-2-K^d-restricted peptides from influenza nucleoprotein are smaller than the corresponding synthetic peptides, which have first been used to determine the respective CTL epitopes[1,3]. As with minor histocompatibility antigens[4], occurrence of viral peptides seems to be heavily dependent on MHC class I molecules, because infected H-2^d cells do not contain the H-2-D^b-restricted peptide, and infected H-2^b cells do not contain the H-2-K^d-restricted peptide. Our data provide direct experimental proof for the above notion on MHC-associated viral peptides on virus-infected cells.

Influenza virus strain A/PR/8/34 was used to infect EL4 (H-2^b) or P815-TR (H-2^d) mouse tumour cells, respectively. The virus-infected cells were subjected to an acid elution procedure that has been used to isolate naturally processed minor histocompatibility antigens[4,5]. Molecules with relative molecular masses less than 5,000 (M_r<5K) contained in the resulting complex mixture were separated by reversed-phase HPLC (Fig. 1). Individual fractions were then tested for recognition by CTL specific for the dominant influenza virus epitopes, which are contained in amino-acid residues 365–380 from the nucleoprotein (NP; peptide NP365–380) for D^b-restricted CTL, and residues 147–158 from the same protein (NP147–158) for K^d-restricted CTL, respectively[1,3]. Material recognized by D^b-restricted CTL eluted at fraction 22 of the extract from infected, but not uninfected, EL4 cells, whereas none of the fractions was recognized by K^d-restricted CTL (Fig. 1*a–d*). By contrast, D^b-

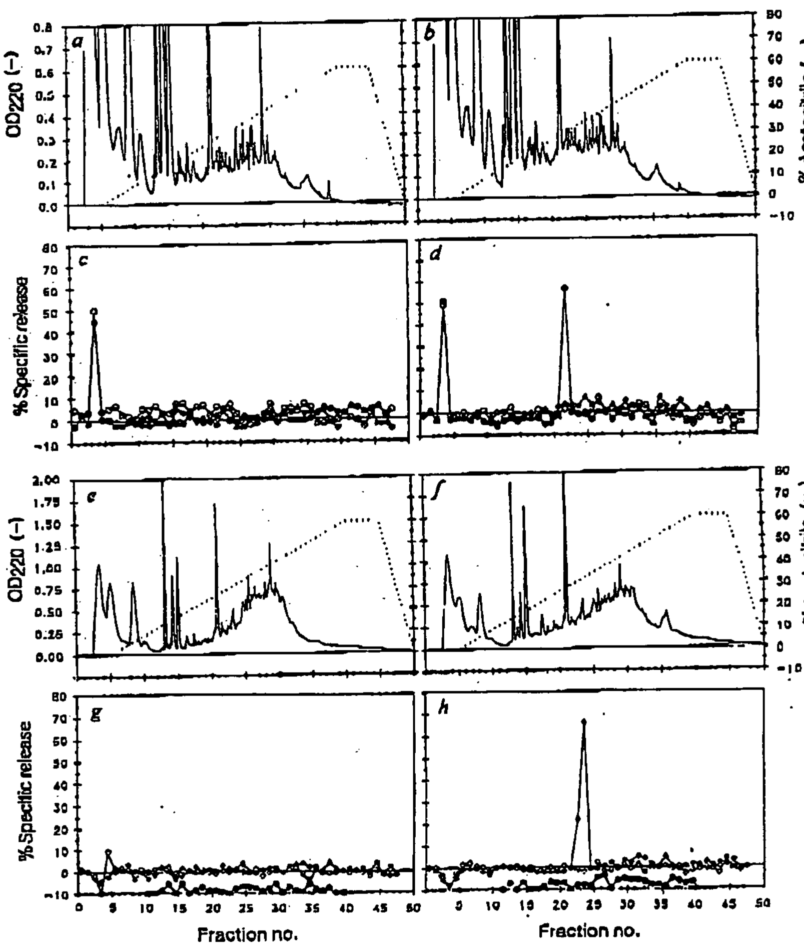

FIG. 1 Isolation of naturally processed viral CTL epitopes from virus-infected cells. EL 4 (H-2^b)(*a–d*) or P815-TR (H-2^d) (ref. 12) (*e–h*) tumour cells (8×10^8) were left untreated (left-hand panels) or were infected with 50,000 units of influenza A/PR/8/34 virus (right-hand panels). Peptides were isolated from cells by acid extraction[4,5] and separated by reversed-phase HPLC (*a, b, e, f*). Solid lines, absorption at 220 nm; dotted lines, percentage of acetonitrile in the gradient. *c, d, g, h*, Individual HPLC fractions were tested for recognition by influenza virus-specific CTL lines LS9 (D^b-restricted, specific for an epitope on NP365–380) (●) or HASI (K^d-restricted, specific for an epitope on NP147–158) (◆) or with medium (○, ◇). Target cells were EL4 cells (○, ●) for LS9 and P815 (H-2^d) cells (◇, ◆) for HASI.

METHODS. Tumour cells were infected as described[6]. Cells were suspended in 0.1% trifluoroacetic acid (TFA), dounced, sonicated and centrifuged as described for spleen cells[5]. Material in the supernatant of M_r>5,000 was removed by gel filtration (G25 Sepharose, Pharmacia). The remainder of the supernatant was separated on a reversed-phase HPLC column (Superpac Pep S, Pharmacia LKB) in 0.1% TFA using a gradient of increasing acetonitrile concentration. Flow rate, 1 ml min^{-1}; fraction size, 1 ml. Individual fractions were collected, dried, resuspended in PBS, incubated with ^{51}Cr-labelled tumour cells (either EL4 or P815) and tested for recognition by influenza-specific CTL in a standard ^{51}Cr release assay as described[5]. Spontaneous ^{51}Cr release of target cells ranged between 15 and 25%. Effector to target ratio ranged between 5:1 to 20:1. CTL line HASI was produced by stimulating spleen cells of a BALB/c mouse (preimmunized with 50 units of A/PR/8/34 virus) with 100 ng ml^{-1} of NP147–158 peptide in minimum essential alpha medium containing 10% FCS, β-mercaptoethanol, glutamine, and antibiotics at 37 °C, 5% CO_2, followed by weekly stimulation with irradiated (33 Gy) syngeneic spleen cells and peptide in medium supplemented with interleukin-2. This CTL line efficiently lyses A/PR/8/34-infected P815-TR, but not EL4 cells. The CTL line LS9 was produced by stimulating spleen cells of a (C57BL/6×DBA/2)F1 mouse preimmunized with a synthetic lipopeptide vaccine[8] containing NP365–380 according to the protocol used to produce HASI. LS9 efficiently lyses EL4 cells infected with A/PR/8/34, but not infected P815-TR cells. Another D^b-restricted CTL line produced by immunization *in vivo* with virus showed recognition patterns of both natural and synthetic peptides identical to that of LS9 (not shown).

restricted CTL did not recognize any fraction from P815-TR extracts, whereas K^d-restricted CTL recognized fraction 24 from infected, but not from uninfected P815-TR cells (Fig. 1*e–h*). We conclude that naturally processed viral peptides exist, that they can be isolated from infected cells by acid elution, and that these peptides are involved in the MHC class I-restricted antigen-processing pathway, as suggested by their MHC dependency. The latter also excludes the possibility that the isolated peptides are artefacts produced during the extraction procedure. Because EL4 and P815-TR cells differ not only at MHC genes, the present data do not prove MHC dependency of processing ('processing' here means not only the cutting of proteins, but also the further fate of the degradation products). By use of MHC- recombinant and mutant mice, however, we have shown for minor histocompatibility antigens that the outcome of processing is dependent on MHC class I molecules[4].

For their molecular identification, the biochemical behaviour of naturally processed peptides was compared with that of the corresponding synthetic peptides. Figure 2*a*, *b* shows reversed-phase HPLC profiles of preparations of synthetic peptides NP147-158 (TYQRTRALVRTG (single-letter amino-acid code)) and NP365-380 (IASNENMETMESSTLE), respectively. Recognition of individual fractions by the respective CTL is shown in Fig. 2*c*, *d*. Not only the main products were recognized, but also some byproducts of lower M_r (such byproducts in small amounts are common to crude synthetic peptide preparations). This result is consistent with reports that CTL may recognize shorter peptides as well, and sometimes much better than the above peptides (for example, NP366–379)[1,6]. At higher dilution of fractions, D^b-restricted CTL failed to recognize the main peak, but still recognized some other fractions, which contained small to undetectable (according to absorbance at 220 nm) amounts of peptide (Fig. 2*f*). Incidentally, both crude synthetic peptide preparations NP147–158 and NP365–380 contained other peptides of smaller size, which coeluted exactly with the respective natural peptide (Fig. 2*e*, *f*). Thus, the naturally processed virus peptides in both cases are different, most likely smaller, than the respective synthetic peptides reported[1,2] to contain CTL epitopes. Both natural peptides are recognized in a concentration-dependent and MHC class I-restricted manner (Fig. 3*a*, *d*). The shorter synthetic by-products coeluting with the natural peptides, again in both cases, are recognized much better than are the nominal synthetic peptides (Fig. 3*b*, *e*). A truncated peptide in the crude synthetic NP147–158 preparation coeluting with the natural peptide was determined by ion spray mass spectrometry to be TYQRTRALV. A subsequently synthesized peptide according to this sequence (NP147–155) coeluted with the natural peptide (not shown) and was indeed recognized (used as crude material) much better than NP147–158 (Fig. 3*c*), about as well as is TYQRTRALVTG (missing R at position 156) which is recognized 1,000-fold better than NP147-158 (ref. 6; Fig. 3*c*). Thus the natural K^d-restricted CTL epitope of influenza NP is likely to be TYQRTRALV. Under this assumption, comparison of the titration curves in Fig. 3*a*, *b*, *c* allows the calculation of the upper limit of peptide molecules extracted per infected cell to be 1,000. By similar analysis, the natural D^b-restricted peptide coeluted with ASNENMETM (NP366–374), which is recognized 1,000 times better than IASNENMETMESSTLE (manuscript in preparation). Thus, a previously identified optimal D^6-restricted NP peptide (NP366–379) happens to share its N-terminal amino acid residue with the natural peptide[1].

Together our experiments show that virus-infected cells produce small peptides from viral proteins which are recognized by MHC class I-restricted CTL, thereby directly confirming the overwhelming evidence provided by MHC crystallography and by experiments with synthetic peptides and truncated genes[1,2,7]. The data also indicate that the use of synthetic peptides to identify T-cell epitopes may be misleading, as very minor byproducts may be responsible for much of the biological effect.

The results in this and in a previous paper on naturally processed minor histocompatibility peptides[4] show two features of MHC class I-restricted antigen presentation. First, a cell produces and maintains exactly one peptide presented to a given

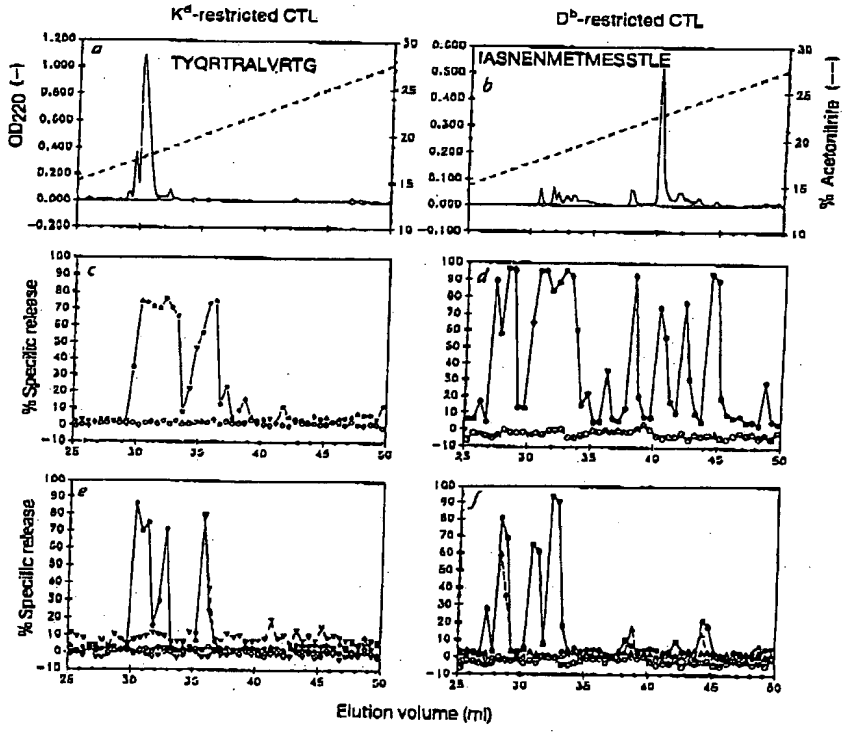

FIG. 2 Naturally processed influenza virus-derived CTL epitopes coelute with synthetic peptides. *a*, Reversed-phase HPLC (at higher resolution as in Fig. 1) elution profiles (solid lines) of synthetic peptide preparations according to NP147–158 (*a*) or NP365–380 (*b*). Dashed lines, percentage of acetonitrile in the gradient. *c*, Recognition of HPLC fractions (diluted 1:2,500) of the NP147–158 preparation by K^d-restricted CTL HASI (◆) or without CTL (◇) using P815 as target cells. *d*, Recognition of fractions of the synthetic NP365–380 preparation at same dilution by D^b-restricted CTL LS9 (●) or without CTL (○) using EL4 as target cells. *e*, Recognition of NP147–158 fractions (diluted 1:62,500) (◆) and of rechromatographed natural K^d-restricted peptide (fraction 24 of Fig. 1*f*, *h*) (▼) by HASI CTL. *f*, Recognition of NP365–380 fractions (diluted 1:62,500) (●) and of rechromatographed natural D^b-restricted peptide (fraction 22 of Fig. 1*b*, *d*) (▲) by LS9 CTL.
METHODS. Peptides were synthesized as described[8]. Peptide preparations (400 μg of each) were chromatographed on the same reversed-phase HPLC column as in Fig. 1, using conditions yielding higher resolution (slower increase of acetonitrile concentration). Flow rate, 1 ml min^{-1}; fraction size, 0.5 ml. The main peaks in *a* and *b* were confirmed by ion-spray tandem-mass-spectrometry to be identical with the nominal peptide sequences, respectively. The fractions identified as naturally processed viral antigens in Fig. 1 were rechromatographed using exactly the same conditions as for the synthetic peptide preparations. Individual fractions were tested for CTL recognition as in Fig. 1.

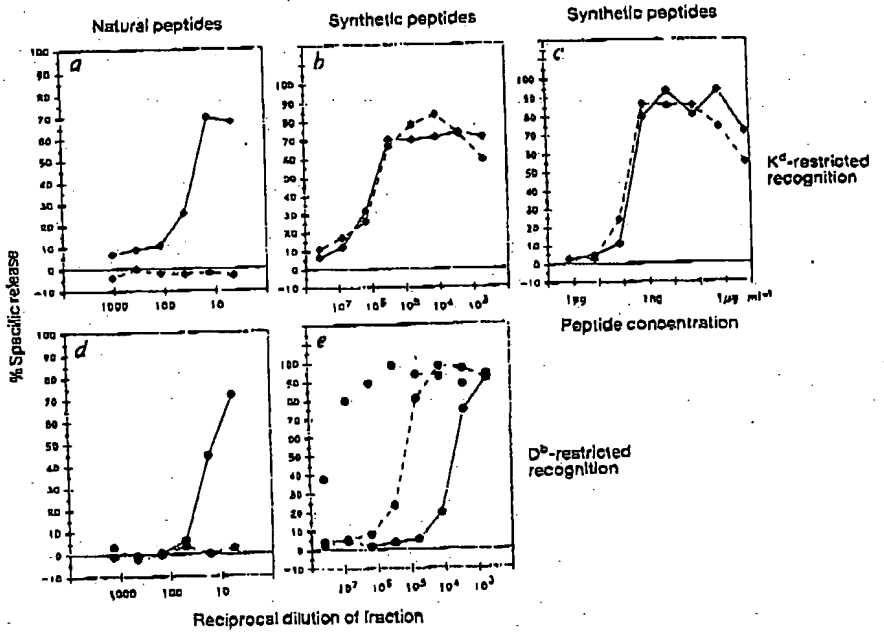

FIG. 3 Titration of natural and synthetic peptide fractions. *a*, Natural K^d-restricted NP peptide (fraction 24 of Fig. 1*f*, *h*) was tested in titrated concentrations for recognition by HASI CTL using P815 (—) or EL4 (- -) as target cells. No killing of EL4 indicates MHC class I-restricted recognition. *b*, Fractions of Fig. 2*a* representing the main synthetic peptide peak (TYQRTRALVRTG; 30.5–31.0 ml elution volume) (—) and the truncated peptide (TYQRTRALV according to tandem-mass-spectrometric analysis) coeluting with the natural peptide (35.5–36.0 ml) (- -) were tested in titrated dilution steps for recognition by HASI CTL. Note the difference between the peptide concentrations of the two samples, as evident from their absorption values in Fig. 2*a*. *c*, Crude batches of synthetic peptide preparations TYQRTRALV (- -) (synthesized on a fully automated simultaneous multiple peptide synthesizer; Zinsser Analytical model 350)[12] and TYQRTRALVTG (—) (missing R at position 156)[8,9] were tested in 10-fold dilution steps for recognition by HASI CTL. *d*, Natural D^b-restricted NP peptide (fraction 22 of Fig. 1*b*, *d*) was titrated into a CTL assay with LS9 CTL using EL 4 (—) or P815 (- -) as targets. *e*, Fractions of Fig. 2*b* representing the main synthetic peptide (IASNENMETMESSTLE; 40.0 to 40.5 ml elution volume) (—), the truncated synthetic peptide coeluting with the natural peptide (28.0–28.5 ml) (- -), and a second truncated, hyper-reactive peptide (32.0–32.5 ml) (· ·) were tested in 10-fold dilution steps for recognition by LS9 CTL.

CTL, as shown by elution of the natural peptide as a single sharp activity peak on reversed-phase HPLC profiles. Second, the processing of endogenous proteins seems to be dependent on MHC class I molecules, as discussed in ref. 4. A database containing many sequences of naturally processed peptides should allow insight into the specificity of the proteases involved, and further our knowledge about the rules of peptide–MHC interactions.

The acid elution method for isolation of naturally processed T-cell epitopes in combination with peptide chemistry including highly sensitive analytical tools such as HPLC and capillary-zone electrophoresis combined with ion-spray tandem-mass-spectrometry should provide straightforward experimental access to the peptide sequences of processed pathogen-derived antigens and has impact on synthetic vaccine design: the efficiency of synthetic peptide vaccines for CTL activation[8,9] could be improved further by using exactly the peptide produced by the infected cells. On the other hand, knowing exactly which peptide is presented naturally to a given T cell could help in the design of prophylactic or therapeutic measures for T cell-mediated autoimmune diseases[10]. □

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Three-dimensional structure of an idiotope–anti-idiotope complex

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SEROLOGICALLY detected antigenic determinants unique to an antibody or group of antibodies[1–3] are called idiotopes. The sum of idiotopes of an antibody constitute its idiotype[3]. Idiotypes have been intensively studied following a hypothesis for the self-regulation of the immune system through a network of idiotype–anti-idiotype interactions[4]. Furthermore, as antigen and anti-idiotypes can competitively bind to idiotype-positive, antigen-specific antibodies, anti-idiotypes may carry an 'internal image' of the external antigen (see refs 5–10 for reviews). Here we describe the structure of the complex between the monoclonal anti-lysozyme FabD1.3 and the anti-idiotopic FabE225 at 2.5 Å resolution. This complex defines a private idiotope consisting of 13 amino-acid residues, mainly from the complementarity-determining regions of D1.3. Seven of these residues make contacts with the antigen, indicating a significant overlap between idiotope and antigen-combining site. Idiotopic mimicry of the external antigen is not achieved at the molecular level in this example.

The crystal structure of the idiotope-anti-idiotope complex FabD1.3–FabE225 (ref. 11) shows the two Fabs roughly aligned along their major lengths, interacting largely through their complementarity-determining regions (CDRs) (Fig. 1*a*). Fourteen residues from the six CDRs of E225, together with a framework residue of V_L, contribute to interatomic contacts with the idiotope (Table 1). In turn, five CDRs and one V_L framework loop contribute 13 residues to the idiotope of D1.3 recognized by E225 (Table 2). FabE225 is centred on V_L of FabD1.3 such that nine residues of the idiotope are located on this domain

Characterization of the Interactions Between MHC Class I Subunits: A Systematic Approach for the Engineering of Higher Affinity Variants of β_2-Microglobulin[1]

Michael J. Shields, Nassim Assefi, Wesley Hodgson, Ellen J. Kim, and Randall K. Ribaudo[2]

Human β_2m (hβ_2m) binds to murine MHC I molecules with higher affinity than does murine β_2m and therefore can be used as a model system to define and dissect the interactions between β_2m and MHC I heavy chains that promote the stability of the complex. In the present study we compare three-dimensional crystal structures of human and murine MHC I molecules and use functional studies of chimeric human:murine β_2m variants to define a region of β_2m that is involved in the higher affinity of hβ_2m for murine MHC I heavy chains. Further examination of the three-dimensional structure in this region revealed conformational differences between human and murine β_2m that affect the ability of an aspartic acid residue at position 53 (D53) conserved in both $\beta_2$ms to form an ionic bond with arginine residues at positions 35 and 48 of the heavy chain. Mutation of residue D53 to either asparagine (D53N) or valine (D53V) largely abrogated the stabilizing effects of hβ_2m on murine MHC I expression in a predictable manner. Based on this observation a variant of hβ_2m was engineered to create an ionic bond between the heavy chain and β_2m. This variant stabilizes cell surface H-2D^d heavy chains to a greater extent than wild-type hβ_2m. Studying these interactions in light of the growing database of MHC I crystal structures should allow the rational design of higher affinity hβ_2m variants for use in novel peptide-based vaccines capable of inducing cell-mediated immune responses to viruses and tumors. *The Journal of Immunology,* **1998, 160: 2297–2307.**

The ability to generate cell-mediated immune responses is critical in the host defense against viral infections and malignant transformation. At the center of these responses is the presentation of viral or tumor-derived Ags to CD8$^+$ T cells by MHC class I molecules. Through a variety of mechanisms, some viruses and tumors have devised ways to prevent the priming of cell-mediated immune responses, in some cases by interfering with the efficient expression of MHC class I molecules on the cell surface (1–9). As the density and half-life of MHC-bound peptides are important in the in vivo priming of cell-mediated immune responses, these effects on expression may serve to block the induction of specific CTL. The half-life of peptide-loaded MHC I molecules on the cell surface can be seconds to days and depends on the affinity of the bound peptide (10). In contrast, empty, or peptide-receptive, molecules are rapidly lost from the cell surface (11–13), ensuring that under normal circumstances these molecules will not acquire exogenously derived peptides.

Although many peptide-loaded class I molecules possess long half-lives, considerable evidence exists demonstrating that there can be substantial exchange of peptide as well as β_2m on the surface of cells in culture (14, 15). In fact, long before the three-dimensional structure of class I molecules was solved, the exchange of endogenous β_2m on murine L cells with bovine β_2m present in the serum added to cell culture medium was reported (16). Although some reports have suggested that peptide ligand exchange appears to occur independently of β_2m exchange (14, 15) or that they are non-co-operative or even antagonistic processes (17), the presence of β_2m in the culture medium is necessary for effective peptide pulsing of cell surface murine class I molecules to occur (18–21). Consistent with this, empty murine MHC I molecules can be stabilized by the addition of exogenous human β_2m (hβ_2m)[3] (12, 13), making them receptive to loading with exogenous peptides and creating stable MHC I complexes that can stimulate CD8$^+$ T cells. Whether the target molecules for these effects of β_2m are pre-existing cell surface molecules that have lost their endogenous peptides, newly emerging peptide-receptive molecules, or even recirculating molecules is not clear. Further, the molecular basis for this effect of hβ_2m remains poorly understood, but probably relies on the greater affinity of human compared with murine β_2m (mβ_2m) for murine MHC heavy chains (22).

Advances in defining the peptide binding motifs of various MHC I molecules, improvements in the ability to predict immunodominant peptide motifs from viral proteins, and sequencing the peptides eluted from the MHC I of isolated tumors provide a basis for the design of peptide-based vaccines. To this end, identifying the molecular nature of the interactions between residues of human or murine β_2m and murine class I heavy chains that contribute to affinity differences will provide the means by which to engineer higher affinity variants of mβ_2m with superior peptide loading ability to be used in animal vaccine models.

Various strategies could be used to create a higher affinity β_2m mutant, including 1) random mutagenesis, 2) interspecies analysis

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[3] Abbreviations used in this paper: hβ_2m, human β_2-microglobulin; mβ_2m, murine β_2-microglobulin; LdE, H-2L^d-transfected E-3 cells; LdE9V, E-3 cell line transfected with H-2L^d containing the E9V substitution.

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of murine and h$\beta_2$ms to define residues and regions that differ and therefore might affect the interaction with MHC heavy chains, and 3) modeling/mutating amino acid residues based on known crystal structures. Random mutagenesis has been conducted previously on hβ_2m to modulate HLA-B27 activity (23). Preparations of randomly mutated hβ_2m were screened for binding to HLA-B27 with a peptide-sensitive mAb and using functional T cell assays. However, due to the nature of the mutagenesis approach, many of the mutants had multiple changes, which complicates the interpretation of the data with respect to the contributions of individual residues. A second method to create a higher affinity β_2m is to take advantage of serendipitous "experiments" of nature. For example, it has been shown that hβ_2m's affinity for murine MHC I heavy chains is higher than that of mβ_2m (22). Since 30 of 99 amino acid residues differ between the two, there are various candidate residues that could be responsible for the higher affinity. The generation of chimeric molecules can assist in localizing the region(s) most likely to account for the affinity difference. Furthermore, individual residues can be changed to determine their specific involvement. Finally, the existing three-dimensional crystal structures can be exploited to predict point mutations in β_2m that could lead to more stable interactions between the MHC heavy chain and β_2m. A combination of the latter two approaches has been taken here to better define the interactions between MHC heavy chains and β_2m and to lay the foundation for the rational design of higher affinity h$\beta_2$ms. Using functional information obtained with human: murine chimeric molecules and an obvious conformational difference in the α carbon trace of human and murine $\beta_2$ms, an individual amino acid residue has been identified that, when mutated to destroy ionic bond(s), caused predictable functional consequences. Furthermore, a gain of function mutant hβ_2m is described that was engineered to create an ionic bond between β_2m and the MHC heavy chain.

Materials and Methods

Chimeric cDNAs and plasmids

RNA transcription plasmids LdCITE and DdCITE were generated by subcloning H-2Ld cDNAs into the transcription vector pCITE 2a (Novagen, Madison, WI). Murine β_2m cDNA was cloned by PCR and inserted into pCITE 2a as previously described (13). hβ_2m cDNA was a gift from Dr. Ken Parker (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and was similarly subcloned into pCITE 2a. The chimeric β_2m constructs HHM (encoding amino acid residues 1–69 of hβ_2m and residues 70–99 of mβ_2m) and MMH (encoding amino acid residues 1–69 of mβ_2m and residues 70–99 of hβ_2m) were generated by taking advantage of a common *Eco*RI site found in both human and murine β_2m cDNAs at the position corresponding to amino acid residue 69. Identical restriction digests of both parent vectors were performed, and the inserts were purified and religated into the complementary vector to generate HHM and MMH.

HMM (encoding amino acid residues 1–34 of hβ_2m and residues 35–99 of mβ_2m) was created using splicing by overlap extension (24) using the splicing oligonucleotide (5′-CAT CCA TCC GAC ATT GAA ATC CAA ATG CTG-3′), which encodes residues 30 to 34 of hβ_2m and residues 35–39 of mβ_2m, and its complimentary oligonucleotide (5′-CAG CAT TTG GAT TTC AAT GTC GGA TGG ATG-3′). MHM was also generated by splicing by overlap extension using HHM in pCITE 2a as template and splicing oligonucleotides that encode residues 30 to 34 of mβ_2m and residues 35 to 39 of hβ_2m (5′-G TTC CAC CCG CCT CAC ATT GAA GTT GAC TTA C-3′) and its complimentary oligonucleotide (5′-G TAA GTC AAC TTC AAT GTG AGG CGG GTG GAA C-3′).

For bacterial expression, human, murine, and chimeric β_2m cDNAs were subcloned from pCITE 2a into pET21-d (Novagen). Specifically, a *Bsp*HI site and initiation ATG were added immediately 5′ of the first codon of the mature β_2m protein by PCR. This full-length PCR fragment was isolated, digested with *Bsp*HI and *Bam*HI (engineered into the 3′ untranslated region), and ligated into pET21-d that had been digested with *Nco*I and *Bam*HI. All chimeric cDNAs were confirmed by sequence analysis using standard techniques.

Site-directed mutagenesis

The hβ_2m cDNA in Bluescript SK (Stratagene, La Jolla, CA) was mutated using the ExSite mutagenesis system (Stratagene) according to the manufacturer's protocol and subcloned into the bacterial expression vector pET-21d(+) (Novagen). The antisense oligonucleotides used for mutagenesis were: D53N, 5′-TAA ATT TGA ATG CTC CAC TTT TTC AAT TCT CTC-3′; D53V, 5′-TAA TAC TGA ATG CTC CAC TTT TTC AAT TCT CTC-3′; and K58E, 5′-TTG TCT TTC AGC GAG GAC TGG TCC TTC-3′. All constructs were confirmed by sequence analysis using standard techniques.

Synthesis and purification of recombinant β_2m

β_2m cDNAs cloned into the bacterial expression vector pET21-d were used to transform BL21 *Escherichia coli*. Cultures (50–200 ml) were grown to an OD_{600nm} of 0.6, and bacterial expression was then induced with 1 mM isopropyl β-D-thiogalactoside. Four hours following induction, bacteria were pelleted and washed in 200 mM Tris-HCl (pH 7.6)/2 mM EDTA and lysed by digestion with lysozyme followed by sonication, and the inclusion bodies were isolated by centrifugation. After washing in cold 200 mM Tris/2 mM EDTA (pH 7.6), the inclusion bodies were solubilized at room temperature for at least 1 h in 3 to 5 ml of 6 M guanidine-HCl containing 0.3 M DTT, 100 mM Tris (pH 8.0), and a mixture of antiproteases (5 μg/ml leupeptin, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), and 1% aprotinin). Following overnight dialysis against 2 l of 6 M guanidine (pH 2.0), the recombinant protein was refolded over 72 h in 0.4 M arginine, 5 mM oxidized glutathione, 100 mM Tris, and 2 mM EDTA at 15°C. Following refolding, the protein was dialyzed exhaustively against PBS at 4°C. Recombinant β_2m was judged to be 80 to 95% pure based on analysis by SDS-PAGE and analytical HPLC. Finally, the protein was concentrated using Centriplus-3 concentrating units (Amicon Corp., Danvers, MA) and purified by preparative fast protein liquid chromatography on a Superdex 75 gel filtration column. Following purification, equivalent β_2m concentrations were calculated based on OD_{280nm} readings.

Transcription of RNA

cDNA clones coding for specific H-2 gene products were linearized 3′ of the cDNA insert by digestion with *Bam*HI. RNA was transcribed from 5 μg of the linearized plasmid with T7 RNA polymerase using the Ribomax T7 RNA transcription system (Promega, Madison, WI) following the manufacturer's protocol. Following transcription, the cDNA template was digested with RNase-free DNase (Promega), extracted with phenol/chloroform/isoamyl alcohol (25/24/1), and precipitated and washed in ethanol.

In vitro translation

RNA was translated using Flexi-Lysate rabbit reticulocyte lysate supplemented with canine pancreatic microsomes (Promega) in a final volume of 50 μl containing 50 μCi of [^{35}S]methionine (SJ.1015, Amersham, Arlington Heights, IL) and 100 mM KCl (to optimize translation of RNA containing the encephalomyocarditis virus 5′ untranslated region found in pCITE transcription vectors) following the manufacturer's protocols. Reactions were incubated for 90 min at 26°C, were terminated by addition of 2 vol of ice-cold 0.75 M KCl, 20 mM Tris-HCl (pH 7.6), and 10 mM EDTA, and were placed on ice. Individual RNAs were first titrated to determine the relative amounts of input RNA needed in the translation reaction.

Isolation of microsomes

Aliquots not exceeding 75 μl from the terminated translation reactions were layered onto 100 μl of an ice-cold sucrose cushion (0.5 M sucrose, 20 mM Tris-HCl (pH 7.6), and 10 mM EDTA) and centrifuged in a Beckman Airfuge (Beckman, Palo Alto, CA) at 22 psi (~100,000 × *g*) using an A-100-18 rotor for 15 min. Supernatants were aspirated completely, and pellets were lysed into 50 μl of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.6), 1% Nonidet P-40, 0.5% aprotinin, 0.5 mM AEBSF, 10 μg/ml leupeptin, and 0.03 M iodoacetamide).

Immunoprecipitation and SDS-PAGE

Solubilized microsomes (10–15 μl) were aliquoted into tubes containing purified mAbs (150 μg/ml final concentration) in lysis buffer containing 3% OVA (Sigma Chemical Co., St. Louis, MO) in a final volume of 100 μl. Tubes were incubated on ice for 1 h, and 30 μl of protein A-Sepharose was added in lysis buffer. Samples were incubated on a rotator for 30 min at 4°C. Protein A-Sepharose was then pelleted by centrifugation at 12,000 × *g*, and supernatants were removed by aspiration. Pelleted beads were then washed three times with 1 ml of lysis buffer at 4°C. Finally,

pelleted washed beads were resuspended in sample loading buffer (0.05 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 μM 2 ME, and 0.01% bromophenol blue), heated in a boiling water bath for 5 min, and electrophoresed on 12.5% SDS-polyacrylamide gels. Gels were subsequently fixed in methanol (7%, v/v)/acetic acid (5%, v/v)/glycerol (10%, v/v), soaked in Enlightning (New England Nuclear-DuPont, Boston, MA), dried, and evaluated by autoradiography. Kodak XAR-5 film (Eastman Kodak, Rochester, NY) was exposed for varying times at −80°C using enhancing screens (DuPont, Wilmington, DE).

Quantitative autoradiography

Exposed films were developed using an X-OMAT automated developer (Kodak). All autoradiographs were evaluated quantitatively by densitometry. Reference to quantities immunoprecipitated in *Results* and *Discussion* derives from such scans. Quantitative densitometry was performed using a Molecular Dynamics scanning densitometer (Sunnyvale, CA) equipped with ImageQuant software (Molecular Dynamics). Quantitation of autoradiographs was conducted on multiple exposures representing different intensities to ensure that scans were within the linear response range of the film. Autoradiographs selected for reproduction in the figures do not necessarily represent the same exposures that were used in densitometry. Calculations of molar ratios in immunoprecipitates were adjusted for the number of methionine residues in the MHC heavy chains, hβ_2m and mβ_2m, respectively.

Cell lines and Abs

L cells (DAP-3) and the TAP-1 defective cell line E-3 (EE2H3) (25) were transfected by the calcium phosphate method with H-2L^d H-2D^d, and H-2L^{d}E9V encoding expression plasmids as previously described (13, 26). B4.2.3 is an H-2D^d-restricted murine T cell hybridoma that is specific for the HIV gp160 envelope protein-derived peptide, p18-I-10 (RG PGRAFVTI) (27). Cells were propagated in DMEM augmented with 10% FCS, 20 mM HEPES, 2 mM L-glutamine, 1% nonessential amino acids, 1% Pen-strep (Biofluids, Rockville, MD), and 0.04 mg/ml of gentamicin sulfate. SKT 4.5 is a DAP-3 cell line stably transfected with genomic H-2D^d (28). The mAbs 28-14-8 and 34-2-12 recognize the α3 domains of H-2L^d and H-2D^d, respectively. The presence of this domain is generally regarded as representative of the total number of molecules, as its formation is independent of association with either β_2m or peptide (29). The mAbs 30-5-7 and 34-5-8 recognize the α2 domains of H-2L^d and H-2D^d, respectively, which typically depend on both association with β_2m and peptide. All mAbs were provided by Dr. David Margulies (National Institute of Allergy and Infectious Diseases, National Institutes of Health).

Peptides

Peptides pMCMV (corresponding to residues 168–176 of the murine CMV pp89 early regulatory protein YPHFMPTNL) and p18-I-10 (derived from the HIV type 1 isolate envelope glycoprotein 120, residues 318–327, RG PGRAFVTI) were provided by Dr. David Margulies (National Institute of Allergy and Infectious Diseases, National Institutes of Health). All peptides were purified by reverse phase HPLC and were >95% pure as determined by analytical HPLC.

Effects of exogenous β_2m on cell surface MHC I epitopes

Transfected cells, grown at 37°C with 5% CO_2, were detached from tissue culture flasks by treatment with versene for 2 min and washed with ice-cold serum-free tissue culture medium. Following a 30-min incubation at 37°C, cells were washed and then resuspended in serum-free medium in the presence or the absence of purified recombinant β_2m. The cells were incubated for 2 h at 37°C and then washed with ice-cold FACS buffer (PBS containing 0.2% bovine albumin and 0.025% NaN_3) before staining with conformationally dependent Abs to class I molecules.

Cell surface staining and flow cytometry

Cells were incubated with purified mAbs 30-5-7, 28-14-8, 34-5-8, or 34-2-12 on ice for 1 h, washed with FACS buffer, incubated with FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, Durham, NC), and incubated for an additional 30 min on ice. Cells were then washed and resuspended in 400 μl of ice-cold FACS buffer. Stained cells were treated with propidium iodide and analyzed on a FACScan II analyzer (Becton Dickinson) using CellQuest 1.2 software. Cells were gated for uniform forward and side scatter and negative propidium iodide staining. A minimum of 5000 data points/sample were counted, and all experiments were either performed in triplicate or repeated at least three times. Values are expressed as the mean fluorescence intensity of representative experiments.

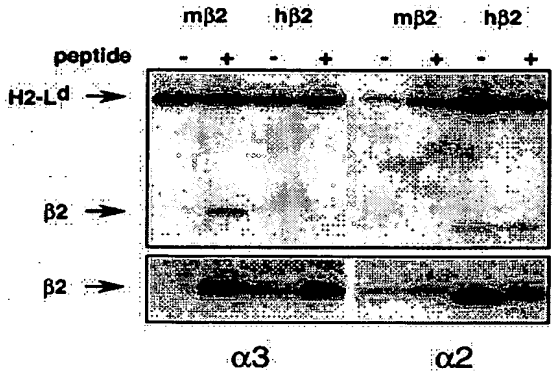

FIGURE 1. In vitro translation and assembly of murine heavy chains with murine and human β_2m. mRNAs encoding H-2L^d were cotranslated with either murine or human β_2m RNA. Microsomes were isolated and solubilized in the presence or the absence of 20 μM pMCMV as indicated. Immunoprecipitation was performed using the indicated Abs and was analyzed by SDS-PAGE. Molar ratios of β_2m to heavy chain were determined by scanning densitometry, taking into account the differences in the number of methionine residues in murine (5) and human (1) β_2m. The *bottom panel* is a longer exposure of the same gel to better visualize the relative intensities of the β_2m bands.

The relative mean fluorescence intensity values varied by <10% between individual replicate experiments.

Growth inhibition studies

The T cell hybridoma growth inhibition was performed essentially as previously described (30). Briefly, H-2D^d-transfected L cells (SKT 4.5) were used as APC to stimulate the T cell hybridoma B4.2.3. APC (2×10^4 cells) were plated overnight in microtiter wells in 10% FCS-containing DMEM at 37°C in 5% CO_2. The next morning, the medium was removed, and the adherent cells were washed three times with PBS and preincubated for a minimum of 1 h at 37°C in 5% CO_2 in serum-free DMEM. The serum-free medium was then aspirated, and the cells were incubated with the indicated concentrations of exogenous β_2m and p18-I-10 (a range of 1×10^{-7} to 1×10^{-13} M) for 2 h at 37°C. After incubation with β_2m and peptide, the cells were washed three times with PBS as described above and incubated overnight with 2×10^4 B4.2.3 cells. The following morning, wells were pulsed with 10 mCi of [^{3}H]thymidine and incubated for 4 h at 37°C. Wells were harvested and counted on an LKB β-Plate scintillation counter (LKB, Rockville, MD). Values were expressed as the percentage of thymidine incorporation relative to that observed in the absence of β_2m and peptide (100%). Calculations of ED_{50} values were performed from the raw data by curve fitting using the Sigmoid logistic $\mathcal{F}(x) = (a - d)/(1 + (x/c)^b + d)$, where a is the minimal plateau value, b is the slope factor, c is the value that results in 50% maximal response, and d is the upper plateau value. Curve fitting was implemented by fixing values a and d based on the raw data, and iterating the logistic 20 to 50 times until no change in b, c, χ^2, or correlation coefficient values were observed. All correlation coefficients were 0.95 or greater.

Results

hβ_2m assembles with murine heavy chains and affects the conformation of the peptide binding domain even in the absence of peptide

We have previously developed an in vitro translation and assembly system to study the generation of murine MHC I molecules (29). Using this system, conditions necessary for stable association of murine β_2m with the heavy chain H-2L^d were established. Initial studies used this model system to explore the nature of the interactions between murine heavy chain and hβ_2m.

Immunoprecipitation of in vitro translated H-2L^d with the mAb 28-14-8 is indicative of a conformed α3 domain and proper intrachain disulfide bond formation (Fig. 1), which is a prerequisite for

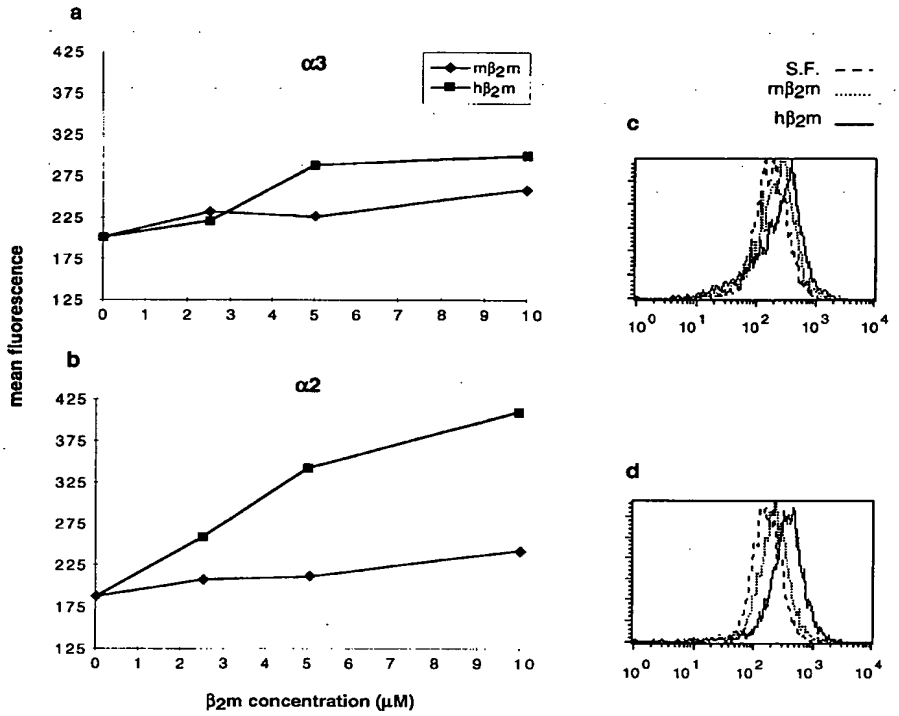

FIGURE 2. The effects of exogenous murine and human β_2m on α3 (*a* and *c*)and α2 (*b* and *d*) domain epitopes on the surface of H-2L^d-transfected L cells (T1.1.1). T1.1.1 cells were incubated for 2 h at 37°C in the presence of the indicated concentrations of β_2m in serum-free DMEM and were then chilled on ice, stained with conformationally sensitive Abs (30-5-7 for the α2 epitope, 28-14-8 for the α3 epitope), and analyzed by flow cytometry. *c* and *d* show FACS profiles for T1.1.1 cells in serum-free medium (—), 10 μM mβ_2m (· · ·), and hβ_2m (—). All values are expressed as the mean fluorescence intensity. In replicate experiments, the relative effects observed varied by <10%.

assembly of in vitro translated MHC I complexes (29). The mAb 30-5-7 recognizes a native α2 domain epitope on H-2L^d that typically depends on presence of β_2m and peptide (29, 31). Consistent with this, 30-5-7 immunoprecipitation of cotranslated H-2L^d and mβ_2m demonstrated a requirement for the presence of an H-2L^d binding peptide for both formation of the peptide binding domain and stable association of the two chains. As has been observed previously, this Ab coprecipitated mβ_2m in a molar ratio of approximately 10:1 (29, 32, 33). In contrast, cotranslation of H-2L^d with hβ_2m resulted in both peptide-independent association of the two chains and generation of a native α2 domain with a molar ratio of heavy chain to hβ_2m of approximately 2:1 in both the presence and the absence of peptide, consistent with a higher affinity interaction between the chains. Calculation of the molar ratio takes into account the fact that mβ_2m has five methionine residues that can be radiolabeled while hβ_2m has only one methionine residue, hence the relatively lighter bands in Figure 1, *lanes 3*, *4*, *7*, and *8*.

Effect of exogenous β_2m on class I cell surface expression

The interaction of mβ_2m with the murine H-2L^d heavy chain is weaker than that with most other murine class I MHC heavy chains, resulting in relatively low total cell surface expression that can be increased by the addition of either hβ_2m or an H-2L^d binding peptide (29, 34). Based on the results shown in Figure 1, one possible mechanism by which hβ_2m exerts this effect is by inducing a native conformation in the peptide binding domain of cell surface molecules, which is generally believed to be critical for cell surface stability (35, 36).

To further explore these interactions, the H-2L^d-transfected L cell T1.1.1 was incubated with graded concentrations of human and murine β_2m in serum-free medium for 2 h, and cell surface expression was determined by flow cytometry (Fig. 2). As generation of the membrane-proximal α3 domain epitope occurs in the endoplasmic reticulum and is independent of association of the heavy chain with either peptide or β_2m, quantitation of this domain reflects the total amount of these molecules on the cell surface regardless of the state of their peptide binding α2 domain (29). Addition of exogenous mβ_2m had a small, but reproducible, effect on both total levels of H-2L^d on the cell surface (as determined by presence of the α3 epitope) and on the α2 domain epitope. hβ_2m had a more pronounced effect on the level of total cell surface H-2L^d compared with effects observed with mβ_2m and an even more dramatic effect on the folding of the α2 domain epitope, again consistent with its higher affinity association with murine heavy chains.

Any effects of exogenous β_2m on cell surface MHC I levels must be observed over the background of endogenous peptide-loaded natively folded molecules on the surface of normal cells. To eliminate this background, we used cell lines with a defect in the peptide transport system (TAP). The murine system allows us to take advantage of a unique property of murine MHC I expression. Unlike human MHC I, which are retained in the endoplasmic reticulum in TAP-negative cells, murine class I molecules accumulate on the cell surface when incubated at reduced temperature or in the presence of peptide and β_2m (37).

H-2L^d-transfected E-3 cells (LdE), which have a regulatory defect in the synthesis of one of the TAP components (13, 25, 38), were treated with increasing concentrations of murine or human β_2m, and the effects on both total (α3 domain) and α2 domain conformed molecules were determined. hβ_2m was more effective than mβ_2m in the generation of both total and α2 conformed MHC I molecules (Fig. 3). Moreover, its effects on the α2 domain epitope were again more pronounced than those on the α3 domain epitope. This suggests that hβ_2m has a qualitatively different effect with regard to the conformation of the α2 domain than does mβ_2m, and this is apparently independent of peptide. This is consistent

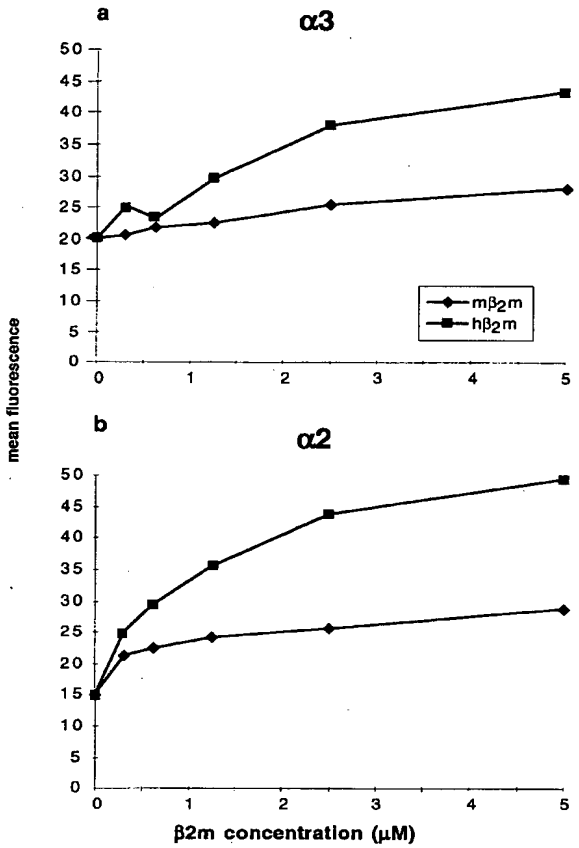

FIGURE 3. The effects of exogenous murine and human β_2m on α3 (*a*) and α2 (*b*) domain epitopes on the surface of LdE. Cells were incubated for 2 h at 37°C in the presence of indicated concentrations of β_2m in serum-free DMEM and were then chilled on ice, stained with conformationally sensitive Abs (30-5-7 for the α2 epitope of H-2L^d, 28-14-8 for the α3 epitope of H-2L^d), and analyzed by flow cytometry. All values are expressed as the mean fluorescence intensity.

with the observations with the in vitro translation assay (Fig. 1) and is indicative of hβ_2m's ability to natively fold this domain in a peptide-independent fashion.

In vitro translation and assembly of chimeric human:murine β_2m with murine MHC I heavy chains

To identify the regions of hβ_2m responsible for the observed effects, we engineered two chimeric human:murine β_2m cDNAs, taking advantage of a conserved restriction site in the cDNAs of both human and murine β_2m. The abilities of these chimeric proteins to assemble with H-2L^d were evaluated by in vitro translation and assembly, focusing on the peptide-independent ability of hβ_2m to generate a native α2 domain. The chimeric β_2m containing the first two-thirds of hβ_2m (HHM) conformed the α2 domain to a similar extent as that observed with native hβ_2m (Fig. 4). Chimeric β_2m consisting of the first two-thirds murine and the last third human (MMH) was indistinguishable from murine β_2m in its effects on α2 domain conformation. Parallel experiments were performed with the murine heavy chain H-2D^d, and similar results were obtained (data not shown).

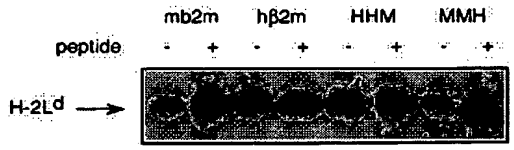

FIGURE 4. Effects of chimeric β_2m on the in vitro translation and folding of H-2L^d. In vitro transcribed RNA was cotranslated with RNAs coding for the indicated chimeric β_2m. Following translation, products were isolated and immunoprecipitated with the α2 domain-specific mAb 30-5-7 and analyzed by SDS-PAGE and autoradiography.

Effects of exogenous chimeric human:murine β_2m on cell surface class I expression

To more precisely determine the regions of hβ_2m responsible for these effects, additional chimeric cDNAs (Fig. 5) were expressed as recombinant proteins and evaluated for their effects on cell surface MHC I expression in TAP-defective cell lines. When LdE cells were incubated for 2 h at 37°C in the absence of β_2m (serum-free medium), H-2L^d expression decreased to approximately 50% of the initial levels in cells grown in 10% FBS (Table I, first two columns). This presumably reflects the inability of the TAP-defective cells to provide peptide-loaded molecules to offset the loss of unstable cell surface molecules that had accumulated in the presence of the bovine β_2m present in FBS. Addition of mβ_2m, HMM (whose first 34 residues are identical with those of hβ_2m), or MHM (which contains only the middle residues (35–69) of hβ_2m) prevented much of the loss of total H-2L^d from the cell surface over 2 h, while addition of hβ_2m resulted in a net increase in total cell surface H-2L^d. Evaluation of the effects of exogenous β_2m treatment on the α2 domain reveals a similar progressive rescue of H-2L^d. HHM was also more effective than either HMM or MHM, suggesting that there were contributions from both regions 1 to 34 and 35 to 69 in stabilizing cell surface H-2L^d. Addition of peptide resulted in dramatic increases in both the α2 conformed and total cell surface H-2L^d, but only in the presence of β_2m, demonstrating that not only can these molecules be preserved

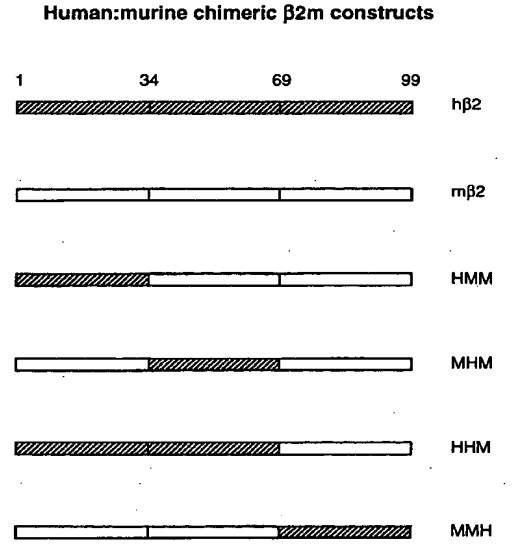

FIGURE 5. Schematic representation of chimeric human (cross-hatched):murine (open) β_2m cDNA constructs.

Table I. *Effects of chimeric β_2m on cell surface expression of H-2L^d in TAP-defective cells*[a]

MHC Molecule	L^d		L^dE9V		D^d	
	α3	α2	α3	α2	α3	α2
Pretreatment	134[b]	120	127	105	108	18
SF	67	65	85	74	106	20
mβ_2m	117	94	130	142	140	48
HMM	110	114	104	134	152	90
MHM	104	101	122	148	134	79
HHM	139	136	188	176	176	100
hβ_2m	172	165	174	213	199	122

[a] H-2L^d-, LdE9V-, and H-2D^d- transfected E-3 cells were incubated in serum-free DMEM for 2 h in the presence of the indicated chimeric β_2m (2.5 μM), and total α3 and α2 domain epitope expression were determined by flow cytometry. Initial levels prior to 2-h incubation in SF medium are indicated (pretreatment).

[b] Mean fluorescence. Background mean fluorescence intensity for all samples was between 10 and 15.

by addition of exogenous β_2m, but they also can be loaded (data not shown).

A number of laboratories have observed that the heavy chains of H-2L^d associate with β_2m with lower affinity than most other murine class I molecules, and the substitution of glutamic acid (E) for valine (V) at position 9 of H-2L^d has been implicated as contributing to this phenotype (13, 32). To determine whether the effects of exogenous β_2m on cell surface H-2L^d were in part due to H-2L^d's lower affinity for β_2m, we tested chimeric β_2m preparations on an E-3 cell line transfected with H-2L^d containing the E9V substitution (LdE9V; Table I). Compared with the 50% loss observed with H-2L^d, there was a 35% loss of total H-2LdE9V from the cell surface during a 2-h incubation in serum-free medium consistent with its higher affinity for β_2m. Addition of either HHM or hβ_2m completely reversed this effect and resulted in an increase in total cell surface H-2L^d of about 35%, while mβ_2m and chimeric β_2m, with only the first or middle regions containing human sequence, had intermediate effects. These effects qualitatively parallel those on the α2 domain of H-2L^{d}E9V, suggesting a tight linkage between the integrity of this domain and stability on the cell surface. Interestingly, the relative effects of hβ_2m on the α2 domain of LdE9V (2-fold increase) were significantly greater than those on the α3 domain (1.4-fold increase). This result is consistent with a greater number of α3 conformed, α2 nonconformed H-2LdE9V molecules on the cell surface than observed with H-2L^d before any β_2m treatment and presumably reflects the higher affinity interaction between the heavy chain and the endogenous β_2m as a consequence of the E9V mutation.

These results suggest that the effects of exogenous hβ_2m on cell surface stability and MHC folding are not unique to low affinity heavy chains such as H-2L^d. To establish that this is a more generalizable phenomenon, we tested the effects of the β_2m chimeras on H-2D^d, a naturally occurring murine MHC I molecule that exhibits higher affinity interactions with β_2m than does H-2L^d (32). LKD8 cells (H-2D^d-transfected E-3 cells) (12) were incubated with chimeric β_2m, total cell surface H-2D^d was determined by flow cytometry with the α3-specific mAb 34-2-12, and the levels of conformed α2 domain epitope were determined with the mAb 34-5-8 (Table I). In contrast to H-2L^d, in the absence of any source of exogenous β_2m, there was no appreciable drop in total cell surface H-2D^d over 2 h, consistent with H-2D^d's higher affinity interaction with β_2m. However, there was little or no α2 domain epitope detectable under these conditions, consistent with these molecules having an unfolded peptide binding domain due to the absence of a functional peptide transporter (TAP). As the half-life of these molecules on LKD8 cells has previously been demonstrated to be only about 10 min (12, 38), their stability over time in serum-free medium probably reflects a steady state between molecules being removed from the surface due to β_2m dissociation and newly emerging molecules with endogenously bound β_2m.

Effects of exogenous β_2m on priming of target cells for T cell recognition

The above data are consistent with exogenous β_2m stabilizing empty class I molecules on the cell surface in a state receptive to loading with exogenous peptide and support the hypothesis that hβ_2m folds the α2 domain even in the absence of peptide. To determine whether the relative effects of the different chimeric β_2m

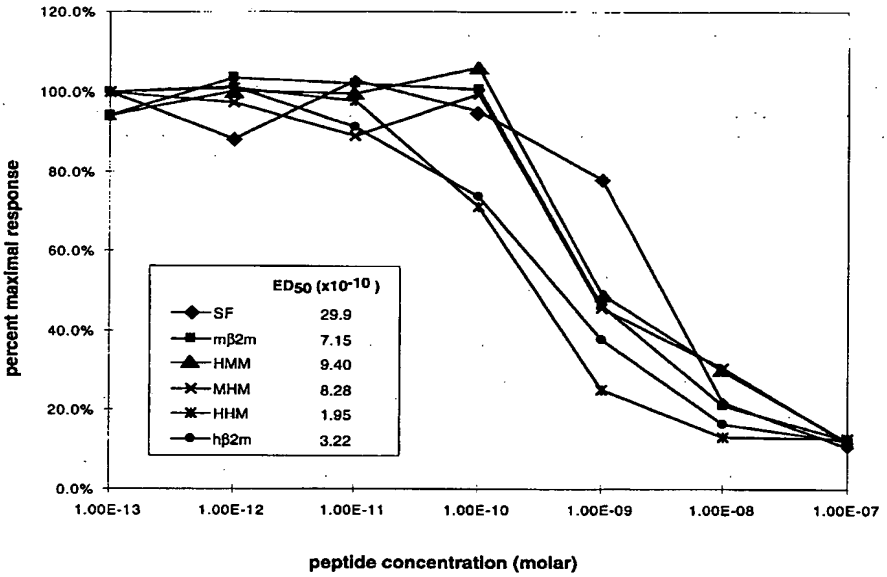

FIGURE 6. Growth inhibition response of the T cell hybridoma B4.2.3 to the H-2D^d-restricted peptide I-10 presented by H-2D^d-transfected L cells. The T cell hybridoma (1×10^5 cells/well) was mixed with the SKT 4.5-presenting cells (2×10^5 cells/well) in the presence of 2.5 μM of the indicated β_2m and incubated overnight at 37°C. Cells were then pulsed with [^{3}H]thymidine, incubated for 4 h, harvested, and counted. Growth inhibition is expressed relative to that observed in the absence of any added APC (maximal response, or 100%), so that complete growth inhibition, which is equivalent to maximal stimulation, would be 0% of the control response. ED_{50} values were calculated from raw data using the Sigmoid logistic as described in *Materials and Methods*. The SEM for all data points was <10%.

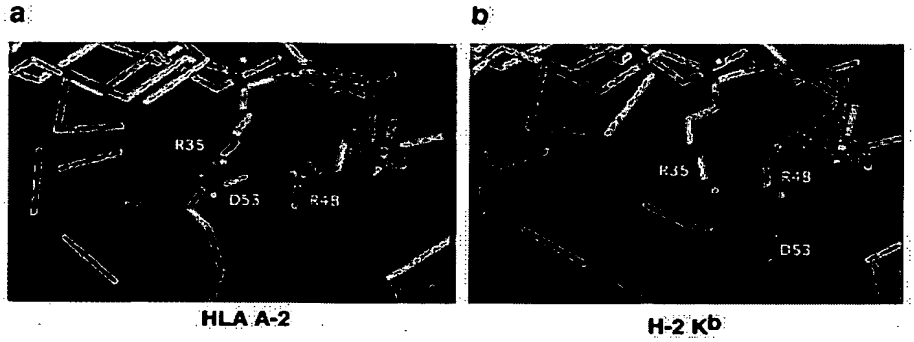

FIGURE 7. Comparison of the x-ray crystal structures of HLA A-2 and H-2K^b showing the interface between β_2m and the floor of the peptide binding groove. Note the negatively charged aspartic acid at position 53 of β_2m (D53) relative to the conserved positively charged arginine residues at positions 35 (R35) and 48 (R48) of both HLA A-2 and H-2K^b heavy chains.

on cell surface class I stability and folding correlate with T cell recognition, we treated the H-2D^d-transfected L cell SKT 4.5 with an H-2D^d binding peptide in the presence of different chimeric forms of β_2m. These cells were then used to stimulate the H-2D^d-restricted, peptide-specific hybridoma B4.2.3, using growth inhibition as a measure of activation (Fig. 6). In the absence of any source of β_2m (SF), the ED_{50} was approximately 3×10^{-9} M peptide. The addition of mβ_2m shifted the sensitivity of the response by about four- to fivefold ($ED_{50} = 7.2 \times 10^{-10}$ M). Consistent with previous results, HMM and MHM resulted in similar increases in the efficiency of peptide loading (ED_{50} = 8.3 and 9.4×10^{-10} M, respectively). However, the addition of either HHM or hβ_2m further improved the effectiveness of peptide loading another three- to fourfold (ED_{50} = 1.9 and 3.2×10^{10} M, respectively), consistent with their ability to stabilize peptide-receptive molecules on the cell surface and make them available for peptide loading.

Structural comparison of hβ_2m and mβ_2m

Our results suggest that the NH_2-terminal two-thirds of hβ_2m contribute to its increased affinity for heavy chain compared with mβ_2m. Alignment analysis of crystal structures for human and murine class I molecules reveal that the S4 strand of hβ_2m (residues 50–56) contains a β bulge, while the same region of mβ_2m is a continuous β strand (22, 39). Superimposing hβ_2m and mβ_2m α carbons reveals that even in this region there is very close alignment, with the notable exception of a single residue at position 53 (D53). The side chain of this residue in hβ_2m lies directly between two conserved arginine residues at positions 35 and 48 in the floor of the heavy chain, with the δ oxygen of D53 coming within 2.9 angstroms of the amino group of R35 and within 4 angstroms of the amino side chain of R48 of the heavy chain (Fig. 7*a*), close enough to form ionic bonds. In contrast, the crystal structures of both H-2K^b (Fig. 7*b*) and H-2D^b (data not shown) reveal that the side chains of D53 lie parallel to the floor of the heavy chain and come only within 4.7 angstroms of the amino group of R35 (H-2K^b) and >6.5 angstroms from R48. To directly assess whether this residue contributes significantly to the interaction between heavy chain and β_2m, we mutated it to either glutamine (D53N) to prevent the formation of ionic bonds or to valine (D53V) to also prevent the formation of hydrogen bonds with other side chains and determined their effects on H-2D^d expression in LKD8 cells (Fig. 8, *a* and *b*). As shown in previous figures, hβ_2m was much more effective in increasing the surface expression of natively folded H-2D^d than was mβ_2m. Significantly, the single mutation of aspartate 53 of hβ_2m to either asparagine or valine resulted in decreased levels of both total and α2 conformed H-2D^d, presumably due to its inability to form ionic bonds with residues in the heavy chain.

The arginine residues at positions 35 and 48 are conserved among most human and murine class I heavy chains. One exception is H-2L^d, which has an arginine at position 35 but has a glutamine at position 48 (40). To determine whether this would result in a less dramatic effect of the D53N or D53V mutations on cell surface H-2L^d than was observed with H-2D^d, we next incubated LdE cells with these mutated forms of hβ_2m. As shown in Figure 8, *c* and *d*, when LdE cells were incubated with recombinant D53N, stabilization of both total and α2 conformed cell surface H-2L^d was as good as or better than that observed with hβ_2m. When these cells were incubated with the D53V form of hβ_2m, whose side chain at position 53 can form neither ionic nor hydrogen bonds with neighboring residues in the heavy chain, a slight decrease in its ability to induce both epitopes was observed compared with that of hβ_2m and D53N. These data establish two important points. First, the D53N mutation did not negatively effect MHC I expression nonspecifically by effecting the folding or secondary structure of the protein, as this mutant worked as well as hβ_2m in stabilizing cell surface H-2L^d, and second, careful analysis of available crystal structures of class I MHC molecules can facilitate the engineering of β_2m variants that predictably affect MHC I surface expression.

Effects of murine β_2m point mutants on cell surface class I expression

A number of "humanized" murine β_2m point mutants have been made by mutating amino acid residues in and around the S4 strand to those found in the human sequence. The only amino acid differences between murine and human β_2m sequences in the S4 strand itself (where D53 resides) are methionines 51 and 54 in the murine sequence, which are histidine and leucine, respectively, in hβ_2m. Therefore, the M51H and M54L double point mutant was made to determine the effects these residues would have on cell surface MHC molecules. Additionally, the two proline residues at positions 33 and 47 in murine β_2m were individually changed to serine and aspartic acid found in hβ_2m (P33S and P47E). When each of these three mutants was assessed for its ability to stabilize cell surface H-2D^d and H-2L^d, no difference was seen relative to wild-type mβ_2m (data not shown).

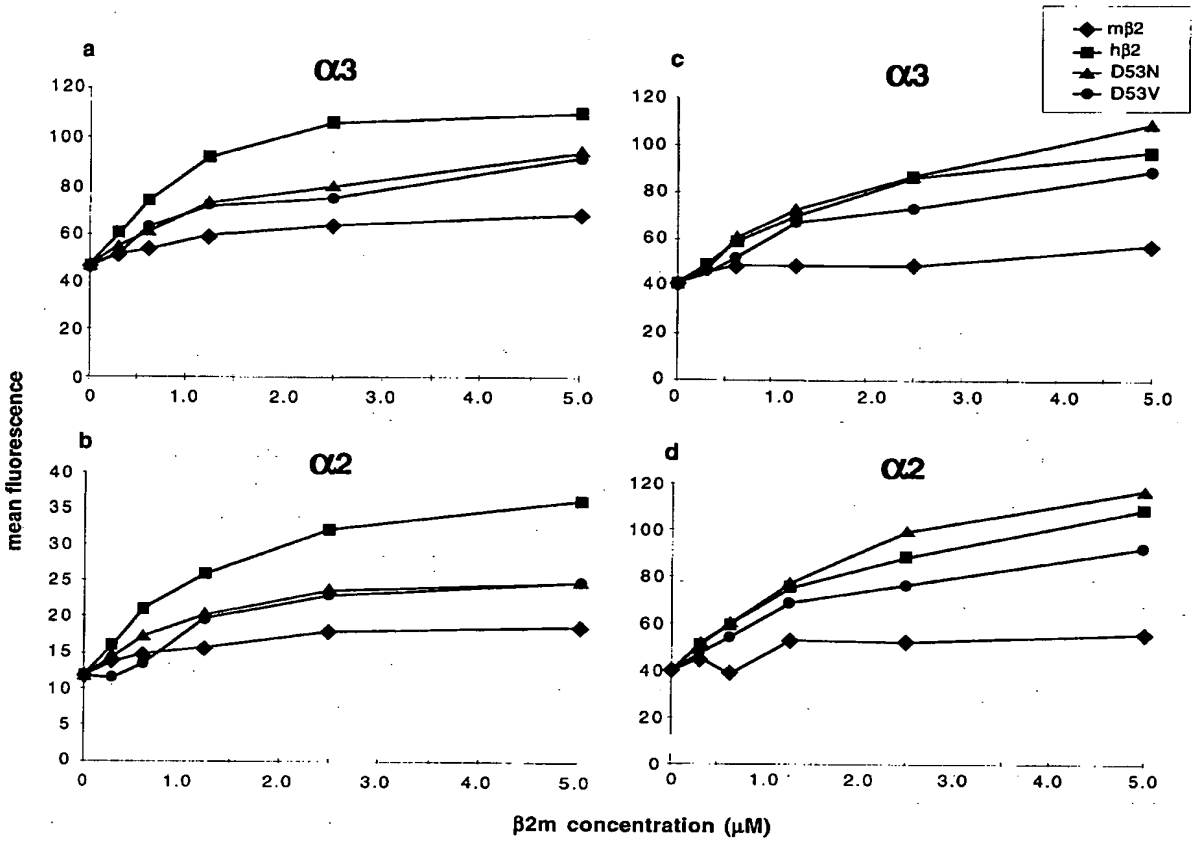

FIGURE 8. The effects of D53N and D53V mutation of hβ_2m on α3 (*a* and *c*) and α2 (*b* and *d*) domain epitope induction of H-2D^d-transfected E-3 cells (LKD8; *a* and *b*) and LdE (*c* and *d*). Cells were incubated for 2 h at 37°C in the presence of the indicated concentrations of β_2m in serum-free DMEM and were then chilled on ice, stained with conformationally sensitive Abs (34-5-8 or 30-5-7 for the α2 epitopes of H-2D^d and H-2L^d, respectively; 34-2-12 or 28-14-8 for the α3 epitopes of H-2D^d and H-2L^d, respectively), and analyzed by flow cytometry. All values are expressed as the mean fluorescence intensity.

Engineering hβ_2m to create an ionic bond

The effects of the position 53 mutation suggested that a single ionic bond may contribute measurably to the stability of heavy chain-β_2m interactions. Upon examination of the crystal structure of HLA-A2, a lysine residue at position 58 of β_2m (K58) was identified that comes in close proximity to a conserved arginine at position 6 of the heavy chain (R6). Therefore, site-directed mutagenesis was used to change the lysine at position 58 of hβ_2m to the negatively charged glutamic acid (K58E) to promote the formation of an ionic bond with R6 of the heavy chain. Since R6 is conserved across not only human but also murine class I molecules, we took advantage of our in vitro cell surface stabilization assay and well-defined mAbs to murine MHC molecules to examine the effect of recombinant K58E on LKD8 cells. Approximately twice the amount of hβ_2m as K58E was required to provide the same level of cell surface H-2D^d stabilization (Fig. 9*a*). The enhanced stabilizing ability of K58E was also demonstrable in β_2m excess (plateau beyond 2.5 μM), suggesting that this effect is qualitatively different from that observed with wild-type hβ_2m and consistent with a higher affinity interaction. The effects on the α2 domain were less dramatic, but consistent with the α3 domain results (Fig. 9*b*). In contrast to H-2D^d, cell surface stabilization of H-2L^d on LdE cells by K58E was essentially indistinguishable from that by hβ_2m (Fig. 9, *c* and *d*). The recently reported crystal structure of H-2L^d (41) reveals that the interatomic distance between the terminal amino groups of the K58 residue of mβ_2m and the R6 of the heavy chain is greater than 11 angstroms, which would make formation of a salt bridge very unlikely. Again these data demonstrate the ability to use structural data to facilitate the engineering of β_2m variants with predictable changes in their functional abilities to stabilize cell surface MHC I molecules.

Discussion

The interaction of hβ_2m with murine heavy chains is qualitatively different from that of mβ_2m

While both mβ_2m and hβ_2m assemble with and promote cell surface loading of class I molecules, hβ_2m is substantially more effective at preserving cell surface class I expression and facilitating peptide loading (Figs. 3, 6, and 8). This property correlates with its ability to induce α2 domain folding even in the absence of peptide, a characteristic not observed with mβ_2m (Fig. 1). The effects of xenogeneic β_2m on murine class I heavy chain conformation have been reported previously (42–45), but the work herein correlates the conformational changes induced on the peptide binding domain by hβ_2m and their effects on the stability of empty molecules.

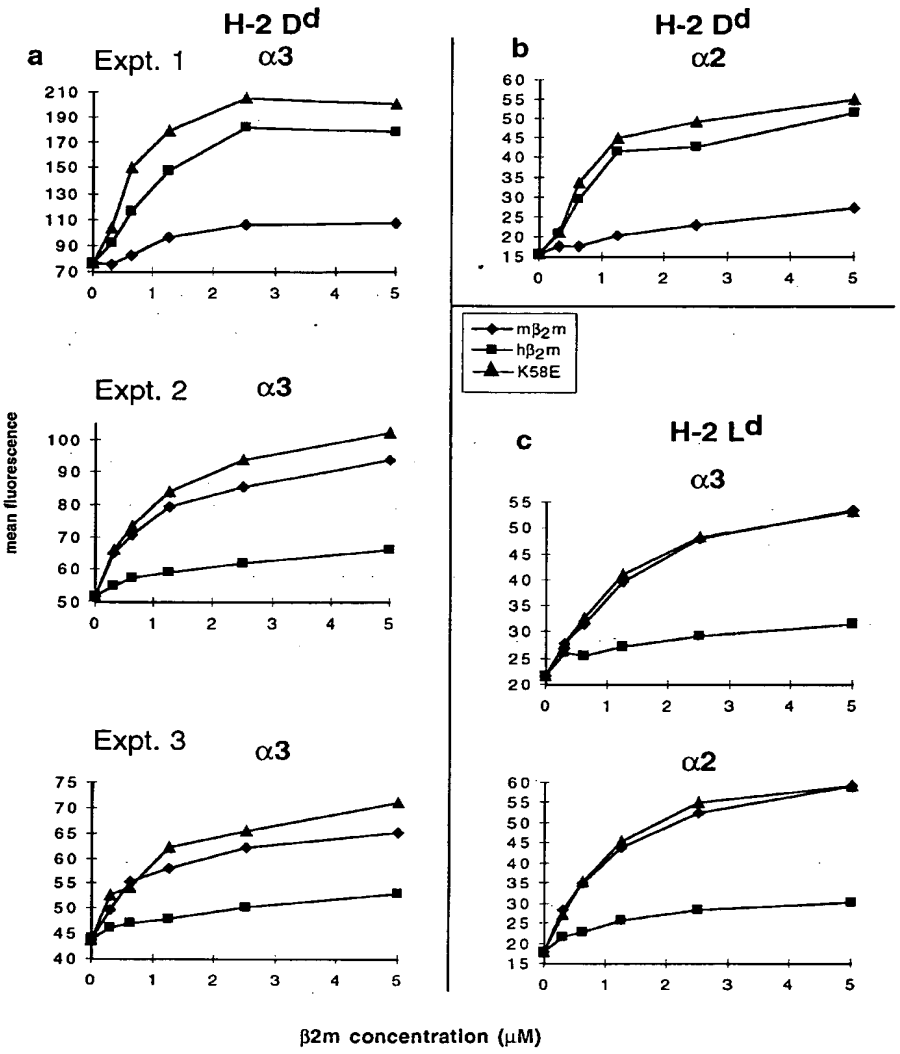

FIGURE 9. The effects of K58E mutation of hβ_2m on α3 and α2 domain epitope induction of H-2D^d transfected E-3 cells (LKD8; *a* and *b*) and LdE (*c*). For LKD8 cells (H-2D^d), results from three replicate experiments are shown. Cells were preincubated in serum-free medium for 30 min, washed, and then incubated for 2 h at 37°C in the presence of indicated concentrations of β_2m in serum-free DMEM. Following incubation, cells were chilled on ice, stained with conformationally sensitive Abs as indicated (34-5-8 or 30-5-7 for the α2 epitopes of H-2D^d and H-2L^d, respectively; 34-2-12 or 28-14-8 for the α3 epitopes of H-2D^d and H-2L^d, respectively), and analyzed by flow cytometry. All values are expressed as the mean fluorescence intensity.

If the ability to conform the peptide binding domain is a general feature of a higher affinity β_2m, it may have important implications for the generation of peptide-based vaccines. For example, it becomes important to determine the T cell response to empty heavy chains that have been stabilized by higher affinity β_2m. These α2 conformed molecules may be recognized as foreign, and therefore determining any effects of these molecules on subsequent immune responses becomes critically important. Recently, Orihuela and colleagues partly addressed this issue in their study of NK cell responses, which are directly affected by MHC I expression. They demonstrated that cell surface H-2D^d molecules on LKD8 cells, stabilized with exogenous hβ_2m in the absence of peptide, did not confer resistance to Ly-49A$^+$ NK cells, while addition of an H-2D^d-restricted peptide did confer resistance (46). Although these experiments were only performed using hβ_2m at relatively low concentrations (17 μg/ml, or 1.4 μM), this would suggest that Ly-49$^+$ NK cells can distinguish between natively folded empty MHC I molecules and peptide-loaded MHC I molecules. Whether this would also be the case with higher concentrations of exogenous hβ_2m remains to be determined. Study of T cell responses to these empty molecules may also provide insight into mechanisms for alloantigen recognition, specifically whether alloreactive T cells require the presence of peptide in the binding groove of class I MHC molecules to become stimulated.

Localizing residues important in the higher affinity of hβ_2m for murine heavy chains

Studies with chimeric murine:human β_2m along with comparative structural analyses focused attention on the S4 strand (residues 50–56). As predicted by these analyses, mutation of aspartic acid at position 53 of hβ_2m to either asparagine or valine significantly affects its ability to stabilize cell surface H-2D^d molecules (Fig. 8, *a* and *b*). This is presumably due to the inability of the mutated residue to form ionic bonds with arginines at positions 35 (R35) and 48 (R48) of the heavy chain and also, in the case of D53V, an inability of the side chain of residue 53 to form hydrogen bonds. Although both hβ_2m and mβ_2m have aspartic acid at this position, residue differences in the neighboring regions may affect the relative orientations of D53.

The fact that the D53N and D53V variants of hβ_2m are still more effective than mβ_2m suggests, not surprisingly, that other interactions also play a role. In this regard, the observations with H-2L^d are particularly significant. hβ_2m is clearly much more effective than mβ_2m in increasing total and α2 conformed cell surface H-2L^d. However, unlike H-2D^d, H-2L^d contains glutamine at position 48, making this residue an unsuitable partner in forming a ionic bond with D53 (26), but a good candidate for forming hydrogen bonds. This may explain why the D53 mutations had much less of an effect on cell surface H-2L^d and why the D53N mutant had slightly better binding. Despite the glutamine at position 48 of H-2L^d, hβ_2m is still more effective than mβ_2m in stabilizing H-2L^d, presumably as a consequence of interactions of other residues in the β_2m:heavy chain interface.

Generation of a higher affinity of hβ_2m for H-2D^d

The mutation of a single residue of hβ_2m (K58E) to promote the formation of a ionic bond improves its ability to fold and stabilize H-2D^d molecules on LKD8 cells (Fig. 9, *a* and *b*). This difference is seen throughout the titration range, most notably after saturation with β_2m, indicating the qualitatively different binding of this mutant. Whether the number or the quality of peptides capable of being stably loaded onto H-2D^d molecules in the presence of K58E is sufficient to influence T cell recognition and stimulation remains to be determined and is currently under examination. The stabilizing effect is not demonstrated with H-2L^d (Fig. 9, *c* and *d*), most likely reflecting the effects of heavy chain polymorphisms. However, since there are no crystal structures of hβ_2m bound to murine heavy chains, it is difficult to determine the structural basis for the difference between H-2D^d and H-2L^d. Considering the above data, another approach for creating higher affinity variants of β_2m could be directed at residues interacting with the α3 domain of the heavy chain, as it is the most conserved of the domains interacting with β_2m. Clearly, it will be important to determine the effects of the K58E and other mutations on human MHC I expression and the ability to facilitate peptide loading on human cells.

Implications for the development of peptide-based vaccines

Until recently, attempts to generate CTL responses in vivo by immunization with peptides have required either traditional adjuvants such as CFA or IFA conjugation of peptides to a variety of lipids or other carriers, or prepulsing of the peptide Ags onto cultured dendritic cells in vitro (47–49). However, in a study by Rock and colleagues, in vivo priming of mice with peptides using only hβ_2m as an adjuvant was convincingly demonstrated (50). The results presented here suggest an underlying mechanism for these observations. Generally, in vivo peptide priming of primary CTL is conducted in the presence of adjuvants that generate local inflammatory reactions that are often associated with increased serum levels of β_2m (51, 52). If the loss of class I molecules from the cell surface is the result of the ordered process of 1) peptide dissociation, 2) β_2m dissociation, and 3) internalization of free heavy chain, then elevated levels of β_2m in inflammatory exudate could stabilize empty or peptide-receptive class I molecules on the cell surface long enough for peptide loading to occur. The addition of hβ_2m to the peptide inoculum provides a means to stabilize these molecules long enough for peptide binding to occur by virtue of its higher affinity interaction with murine MHC heavy chains and thereby generate loaded molecules capable of stimulating CD8$^+$ T cells. Additionally, hβ_2m may have contributed to the in vivo priming by supplying a helper epitope in the form of processed hβ_2m that was represented by MHC class II molecules to CD4$^+$ T cells.

Based on this model, one would predict that the use of syngeneic mβ_2m would not be as effective in promoting in vivo peptide priming in their system unless supplied with a source of T cell help. Considering the data herein, in vivo priming probably would have required significantly higher concentrations of mβ_2m and may not have worked at all. The 30% sequence disparity between hβ_2m and mβ_2m becomes an important consideration in their studies in particular and in the generation of practical vaccines in general. For general vaccine considerations, the likely necessity for multiple rounds of immunization or subsequent immunization with different agents seems to be incompatible with the probable antigenicity of a protein adjuvant with 30% difference in amino acid sequence than the host's own β_2m. Consequently, engineering individual mutations that promote interchain stability, e.g., via the formation of ionic bonds, may lead to the generation of high affinity murine β_2m variants with minimal antigenicity for use in animal vaccination models. Hence, these and subsequent studies are the initial steps to provide a paradigm for the engineering of higher affinity hβ_2m variants for use in peptide-based vaccines in humans.

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